UNIVERSIDAD AUTÓNOMA DE MADRID

Departamento de Bioquímica

CARACTERIZACIÓN CLÍNICA Y GENÉTICA DE FAMILIAS ESPAÑOLAS CON RETINOSIS PIGMENTARIA AUTOSÓMICA DOMINANTE MEDIANTE SECUENCIACIÓN MASIVA Y OTRAS TÉCNICAS DE DIAGNÓSTICO MOLECULAR

TESIS DOCTORAL

Patricia Fernández San José

Madrid, 2017

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TESIS DOCTORAL

Memoria presentada por **Patricia Fernández San José**, licenciada en Farmacia, para optar al título de Doctora por la Universidad Autónoma de Madrid

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CERTIFICAN:

Que la presente memoria titulada "*Caracterización clínica y genética de familias españolas con retinosis pigmentaria autosómica dominante mediante secuenciación masiva y otras técnicas de diagnóstico molecular*", que presenta Dña. Patricia Fernández San José para obtener el Grado de Doctor, ha sido realizada bajo nuestra dirección, autorizándola para su presentación al Tribunal Calificador.

Madrid, 19 de junio de 2017

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Resumen

Las distrofias hereditarias de retina (DR), con una prevalencia de 1: 3000, son la principal causa de discapacidad visual registrada en el mundo desarrollado. Este grupo de patologías están causadas por la degeneración de los fotorreceptores de la retina. Las DR representan una de las enfermedades hereditarias más heterogéneas, tanto clínica como genéticamente. Se han descrito modelos de herencia tanto mendeliana como no mendeliana. Al menos 260 genes han sido identificados como causantes de DR y de ellas, la retinosis pigmentaria (RP) es la forma más común con 80 genes asociados .

Esta tesis doctoral se ha centrado en el estudio de familias españolas afectadas de retinosis pigmentaria de herencia autosómica dominante (adRP), que suponen alrededor del 15 % de todos los casos de RP en España. Se ha realizado un estudio de la implicación del gen *RHO*, el más frecuentemente alterado en RP, en una cohorte de 200 familias presentando una prevalencia de un 21 %. De las 27 mutaciones detectadas, casi un tercio corresponden a mutaciones nuevas no descritas previamente en la literatura, ampliando el conocimiento de este gen en nuestra población. Además, se ha detectado en el 4,5% de nuestras familias la mutación p.Pro347Leu, presentándose como la más frecuente estableciendo para ella estudios de correlación genotipo-fenotipo que ayuden a orientar el diagnóstico y asesorar respecto al pronóstico y posible futuro tratamiento.

Además se han desarrollado diferentes estrategias basadas en tecnología de secuenciación masiva, la primera ha consistido en el uso de un panel de 73 genes con la cual se han caracterizado 16 de 59 familias, obteniendo una tasa de diagnostico del 27%. La siguiente aproximación aplicada fue la secuenciación de exoma completo, que nos ha permitido establecer una nueva asociación fenotípica para el gen *PRPS1*, ampliando el espectro fenotípico asociado al mismo. Además, mediante esta tecnología ,se pudieron reclasificar genética y/o clínicamente tres familias y en una cuarta familia se identificó una gran deleción en el gen *PRPF31* utilizando los datos generados por el exoma, demostrando el gran potencial de esta tecnología para la detección de reordenamientos genómicos.

Los resultados de este trabajo ponen de manifiesto los avances en el diagnóstico de pacientes con enfermedades con elevada heterogeneidad genética y clínica conseguidos gracias a la implementación de la secuenciación masiva, lo que ha conducido a la elaboración de un algoritmo más efectivo concretamente para el diagnóstico de familias con adRP.

Abstract

Inherited retinal dystrophies (IRD), with a prevalence of 1:3000, are the first cause of visual impairment in the developed world. This group of pathologies is caused by degeneration of the retinal photoreceptors. At present, DR represents one of the most clinically and genetically heterogeneous hereditary diseases and both Mendelian and non-Mendelian inheritance models have been described. At least 260 genes have been associated with IRD and Retinitis Pigmentosa (RP) is the most common disease, with 80 genes identified.

This doctoral thesis has focused on the study of Spanish families affected by autosomal dominant retinitis pigmentosa (adRP), about 15% of all RP in Spain. A study of the implication of the *RHO* gene, the most frequently altered in RP, in a cohort of 200 families presenting a prevalence of 21%. Of the 27 mutations detected, almost one third correspond to new mutations not previously described in the literature, increasing the knowledge of this gene in our population. In addition, the p.Pro347Leu mutation has been detected in 4.5% of our families, being the most frequent finding for genotype-phenotype correlation studies that help guide the diagnosis.

In addition, different strategies have been developed based on Next Generation Sequencing technology, the first one consisted in the use of a panel of 73 genes with which 16 of 59 families have been characterized, obtaining a diagnosis rate of 27%. The next applied approach was the whole exome sequencing, which allowed us to establish a new phenotypic association for the *PRPS1* gene, amplifying the phenotypic spectrum associated with this gene. In addition, through this technology, three families could be reclassified genetically and / or clinically and in a fourth family a large deletion in the *PRPF31* gene was identified using the data generated by the exoma demonstrating the great potential of this technology for the detection of genomic rearrangements.

The results of this work show the advances in the diagnosis of patients with diseases with high genetic and clinical heterogeneity achieved through the implementation of Next Generation Sequencing, which has led to the development of a more effective algorithm specifically for the diagnosis of families with adRP.

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Clave de abreviaturas

aCGH *Comparative Genomic Hibridation array*

ACL Amaurosis Congénita de Leber

ADN Ácido desoxirribonucleico

adRP Retinosis Pigmentaria autosómica dominante

ALMS Síndrome de Alström

APEX *Arrayed Primer Extension*

ARN Ácido ribonucleico

ARNm ARN mensajero

arRP Retinosis Pigmentaria autosómica recesiva

ATP Adenosín Trifosfato

AV Agudeza Visual

BBS Síndrome de Bardet –Bield

CV Campo Visual

CNV *Copy Number Variation*

CMTX5 Charcot-Marie-Tooth tipo 5 ligado al cromosoma X

CRISPR *Clustered Regularly Interspaced Short Palindromic Repeats*

CSNB *Congenital Stationary Night Blindness*

DBC Distrofia de Bastones-Conos

DCB Distrofia de Conos- Bastones

DFN2 Sordera neurosensorial no sindrómica ligada al cromosoma X.

DGGE Denaturing gradient gel electrophoresis

DM Distrofia Macular

DMAE Degeneración macular relacionada con la edad

DR Distrofias de retina

EPR Epitelio pigmentario de la Retina

ERG Electrorretinograma

GMP Guanosín monofosfato

CMPc Guanosín Monofosfato cíclico

GDP Guanosín Difosfato

GTP Guanosín Trifosfato

HRM *High resolution melting*

IRD *Inherited Retinal Dystrophy*

Clave de Abreviaturas

LCA *Leber Congenital Amaurosis*

LOF *Loss Of Function*

LORD *Late-Onset Retinal Degeneration*

MAF *Minor Allele Frequency*

MLPA *Multiplex Ligation-dependent Probe Amplification*

NGS *Next generation sequencing*

NMD *Non-mediated ddecay*

PRPH2 Periferina

PRPS1 Phosphoribosylpyrophosphate synthetase 1

qPCR *PCR cuantitativa*

RE Retículo endoplasmático

RHO Rodopsina

RP Retinosis pigmentaria

RT-PCR Reacción en cadena de la polimerasa con transcriptasa inversa

SNV *Single Nucleotide Variant*

SLS Síndrome de Senior Locken

sRP *sporadic Retinitis Pigmentosa*

SSCP *Single-strand conformationpolymorphism*

VREF Vitreorretinopatía exudativa familiar

VUS *Variant of Uncertain Significance* (Variante de Significado Incierto)

WES *Whole exome sequencing*

WGS *Whole genome sequencing*

XlRP *X- linked Retinitis Pigmentosa*

CAPÍTULO I

Introducción

1. La visión

La visión es una de las principales capacidades sensoriales que nos permite interpretar nuestro entorno gracias a los estímulos luminosos que alcanzan el ojo y que se transforman en impulsos eléctricos mediante los fotorreceptores, que son las células especializadas de la retina. El nervio óptico transmite los impulsos eléctricos generados en la retina al cerebro, donde son procesados en la corteza visual y es allí donde tiene lugar el complicado proceso de la percepción visual gracias al cual somos capaces de percibir la forma de los objetos, identificar distancias, detectar los colores y el movimiento.

1.1. Anatomía de ojo humano

Desde un punto de vista morfológico el ojo está constituido por el globo ocular, estructuras anejas (párpados, conjuntiva, aparato lacrimal) y vías ópticas. El globo ocular lo constituye una esfera cuya pared está formada por tres capas concéntricas superpuestas (Figura 1): i) **Capa fibrosa externa** comprendida por la esclerótica y la córnea; ii) **Capa vascular media** o úvea formada por la coroides, el cuerpo ciliar y el iris; iii) Capa nerviosa interna o **retina**. La estructura anatómica afectada, en dónde tienen lugar las enfermedades oftalmológicas hereditarias estudiadas en esta memoria, es la retina.

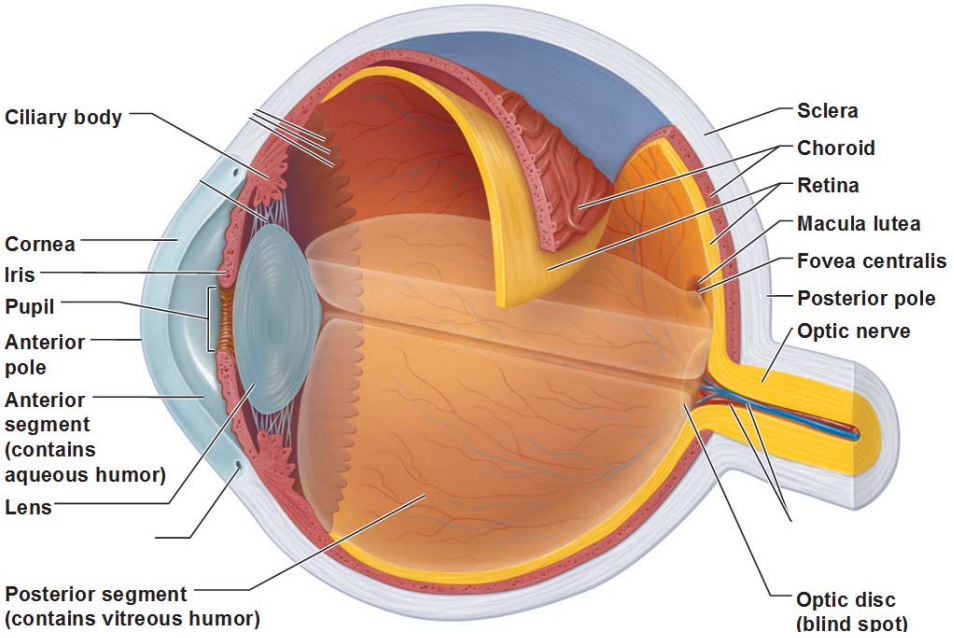

Figura 1. Anatomía del ojo humano.

1.2. Función, desarrollo y estructura de la retina

La función principal de la retina es transformar la luz que incide en este tejido en una serie de fenómenos químicos y eléctricos que finalmente se traducen en impulsos nerviosos que son enviados hacia el cerebro a través del nervio óptico. La retina es una parte del sistema nervioso central que deriva del tubo neural. Su desarrollo comienza en estadíos tempranos del desarrollo embrionario dando lugar a una estructura en forma de capas que dan un aspecto estratificado formado por diferentes tipos celulares (Figura 2).

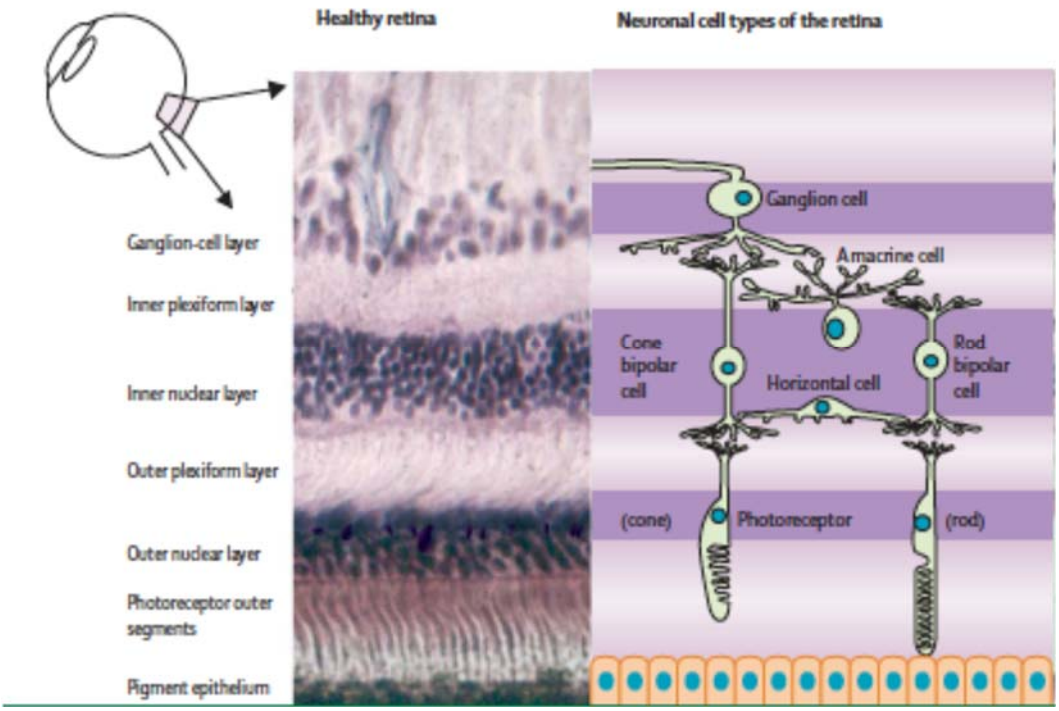

Figura 2. Estructura de la retina humana. A) Sección de retina humana formada por diferentes capas. B) Representación de los distintos tipos celulares. Imagen adaptada de Hartong, *et al*., 2006.

El **epitelio pigmentario de la retina** (EPR) está formado por células epiteliales que se encuentran en contacto con el segmento externo de los fotorreceptores a través de sus microvellosidades. Sus funciones principales son el aporte de nutrientes, reciclaje de los discos de los segmentos externos de los fotorreceptores, secreción de factores de crecimiento y participación en el ciclo visual. En la **capa limitante externa** se encuentran los segmentos externos de los fotorreceptores. La **capa nuclear externa** está constituida por los núcleos de los fotorreceptores, es avascular y dependiente de los coriocapilares. En la **capa plexiforme externa** tiene lugar la sinapsis entre los axones de los fotorreceptores y las dendritas de las células bipolares. La **capa nuclear interna** contiene los núcleos de células bipolares, amacrinas,

horizontales y células gliales de Müller; en la **capa plexiforme interna** se encuentran los axones de las células bipolares, amacrinas y ganglionares. Y, por último, la **capa celular ganglionar**, formada por los cuerpos celulares de las células ganglionares, cuyos axones formarán el nervio óptico. También contiene células amacrinas y vasos sanguíneos retinianos.

1.3. Fotorreceptores

Son células fotosensibles que se encuentran en la retina. Su estructura consta de un **segmento externo**, donde se localizan los pigmentos visuales y comienza el proceso de fototransducción, un **segmento interno** con funciones en la síntesis y metabolismo de proteínas, un **cilio conector**, el **núcleo** y unas **terminaciones sinápticas** que conectan con las células horizontales o bipolares. (Figura 3).

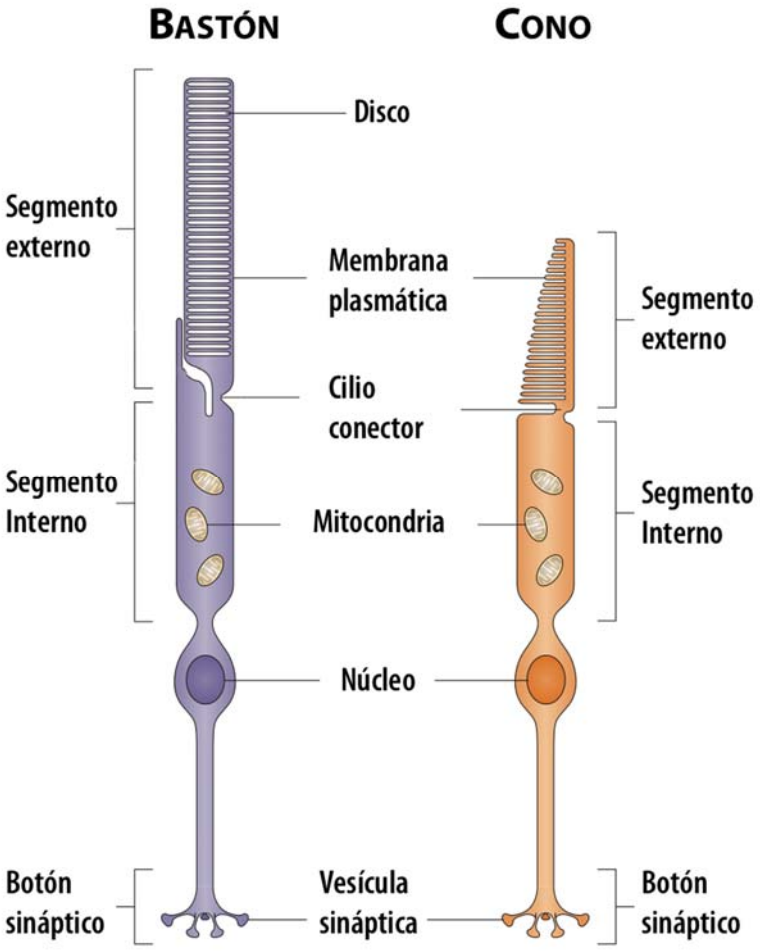

Figura 3. Esquema de los tipos de fotorreceptores de la retina. Imagen adaptada de Wright, *et al*., 2010.

Existen principalmente dos tipos, los bastones y los conos. Los **bastones**, que representan el 95% de los fotorreceptores, aproximadamente 120 millones (Kolb *et al*., 1995) y que se encuentran distribuidos por toda la retina excepto en la fóvea. Con mayor densidad a medida que nos alejamos de ella, son los responsables de la visión periférica, además son altamente sensibles a la luz, por lo que están implicados en la visión nocturna (visión escotópica). En sus discos se encuentra un único tipo de pigmento, la rodopsina, que es la responsable de la visión en blanco y negro. Está formada por una proteína transmembrana llamada opsina que se une covalentemente al retinal, que es un cromóforo derivado de la vitamina A. La opsina es una cadena polipeptídica formada por unos 348 aminoácidos que consta estructuralmente de tres dominios, un dominio citoplasmático que se corresponde con el extremo C-terminal, donde se produce la transducción de la señal luminosa; un dominio transmembrana que consta de 7 hélices que atraviesan perpendicularmente la membrana celular y el dominio extracelular que se corresponde con el extremo N-terminal (Figura S1). El retinal es la parte sensible a la luz y está unido a una de las hélices en el centro de la molécula y colocado perpendicularmente. Presenta dos conformaciones, en la oscuridad el retinal se encuentra en la forma *cis*, pero cuando un fotón de luz es absorbido, rápidamente cambia a la forma *trans*, variando no solo la conformación del retinal sino también de la opsina.

El otro tipo de fotorreceptores son los **conos**, representando el 5%restante, en torno a 6 millones. Se localizan en la mácula y en mayor densidad en la fóvea, donde cada cono está conectado a una célula bipolar, por lo que será la zona de mayor agudeza visual. Además tienen poca sensibilidad, por lo tanto se activarán con luz diurna. Existen tres tipos de conos con distintos pigmentos, el rojo, verde y azul, que absorben para diferentes longitudes de onda y que confieren la capacidad de visión cromática.

1.4. Fototransducción

Es el proceso mediante el cual los fotorreceptores, en respuesta a su activación por un fotón, generan un impulso nervioso. Los pigmentos visuales van a activar una cascada de reacciones enzimáticas y bioquímicas como respuesta a la luz (Figura 4), que finalmente producirán impulsos nerviosos que se transmitirán al cerebro. (Chabre & Deterre, 1989; Arshavsky*et al*., 2002).

Se compone de 2 fases, una luminosa y otra oscura. En la **fase luminosa** la rodopsina absorbe un fotón de luz y provoca la isomerización de la molécula retinal, produciéndose un cambio conformacional de la rodopsina a su estado activo y cataliza el intercambio de los

nucleótidos GDP (Guanosil difosfato) por GTP (Guanosil trifosfato) de una proteína G denominada transducina que consta de 3 subunidades (α, β y γ). La subunidad α tiene una función activadora de la fosfodiesterasa (PDE) que va a estimular la degradación de una molécula de GMPc (monofosfato cíclico de guanosina). Por tanto, en presencia de luz y como consecuencia de la activación de la PDE, los niveles de GMPc disminuyen ocasionando el cierre de canales de Na^+, que se acumula en el exterior de la membrana plasmática provocando su hiperpolarización y el cierre de los canales de calcio dependientes de voltaje, lo que a su vez conlleva una disminución de la entrada de Ca^{2+} en la sinapsis. El resultado final es una disminución de la secreción del neurotransmisor glutamato por parte de los fotorreceptores que dejará de inhibir a las células bipolares formándose un impulso nervioso que es transmitido a las células ganglionares y de éstas al cerebro.

Durante la **fase oscura** aumentan los niveles de GMPc (GMP cíclico) provocando la apertura de canales de Na^+ que despolarizan el fotorreceptor. Posteriormente los canales de Ca^{2+} se abrirán con la consiguiente entrada de estos iones en el interior celular, permitiendo la liberación continua del neurotransmisor glutamato.

1.5. Cascada de inactivación

Para devolver el fotorreceptor a su estado de reposo, cada reacción de la cascada catalítica activada por la luz debe estar compensada por una reacción de inactivación que se lleva a cabo mediante la fosforilación de la rodopsina por la rodopsina quinasa, seguida de la posterior unión de la arrestina, codificada por el gen *SAG,* y de la inactivación de la PDE mediante la hidrólisis de GTP unido a la subunidad α-GTP de la transducina junto con la unión de un complejo de multiproteínas denominado RGS9-1.Gα 5.R9AP (Hu & Wensel, 2002). Por otro lado la restauración de los niveles de GMPc citoplasmático se lleva a cabo por medio de una enzima denominada guanilato ciclasa que se encarga producir GMPc a partir de GTP, cuya actividad enzimática está estimulada por las proteínas activadoras de la guanilato ciclasa, que detectan el descenso de Ca^{2+} secundario al cierre de canales.

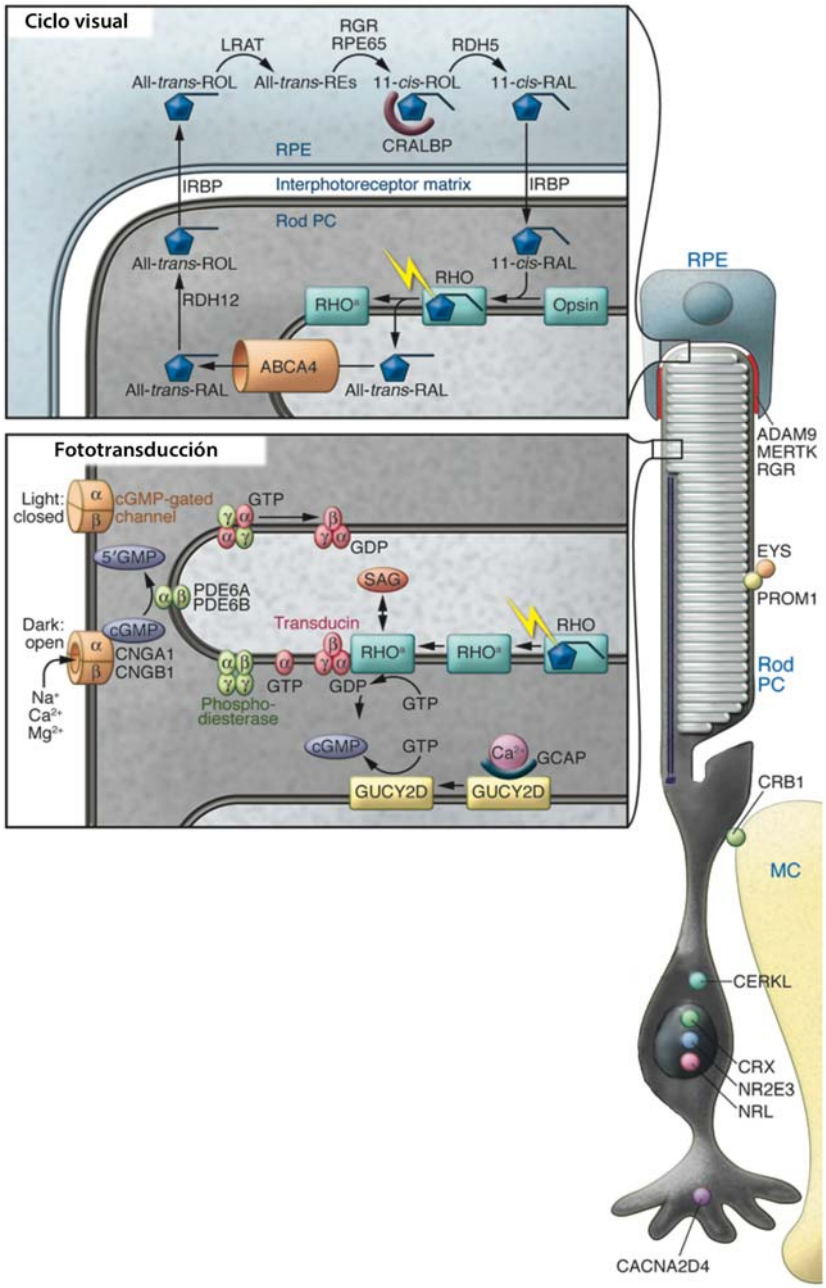

Figura 4. Esquema de la fototransducción y el ciclo visual. Imagen adaptada de Hollander*et al*., 2010.

1.6. Ciclo visual

Es el proceso por el cual los pigmentos visuales inactivos se regeneran para mantener activos los fotorreceptores. Cuando la rodopsina se inactiva por fosforilación, la posterior unión a la arrestina provoca su descomposición, generándose la opsina y el *todo-trans*-retinal que será transportado al EPR a través de la proteína transportadora ligante de ATP (ABCA4). Posteriormente, el *todo-trans*-retinal se convierte en *11-cis*-retinal que vuelve a los bastones para unirse a la opsina libre y regenerar el pigmento visual (Figura 4).

2. Distrofias hereditarias de retina

Las Distrofias de Retina (DR) son un conjunto de enfermedades causadas por la afectación primaria de los fotorreceptores. Su prevalencia es de 1:3000 por lo que son consideradas como enfermedades "raras" o poco frecuentes. Son de carácter hereditario, presentan una extremada heterogeneidad clínica y genética, tienen una evolución generalmente progresiva y no tienen tratamiento en la actualidad (Ayuso & Millan, 2010). Aunque en estadíos avanzados de la enfermedad existe afectación de ambos tipos de fotorreceptores, las distrofias de retina se pueden clasificar según el tipo de fotorreceptor que se ve afectado de forma primaria en **formas centrales** y **formas periféricas**. Las primeras se caracterizan por una degeneración primaria de los conos, con una pérdida de agudeza visual como síntoma inicial, alteración en la respuesta de los conos y alteración en la percepción de los colores. Según su evolución, pueden distinguirse **formas estacionarias** como la acromatopsia completa e incompleta y monocromatismo de conos azules y **formas progresivas** donde, en estadíos posteriores, pueden verse afectados también los bastones. Ejemplos de este grupo son algunas Distrofias Maculares (DM), Distrofia de Conos (DC), Distrofia de Conos-Bastones (DCB) y algunos tipos de Amaurosis Congénita de Leber (*Leber Congenital Amaurosis, LCA*).

Las **formas periféricas** se caracterizan por una distrofia primaria de los bastones, con ceguera nocturna como síntoma inicial en la mayoría de los casos, pérdida del campo visual periférico y alteración en la respuesta eléctrica de los bastones. Según su evolución, pueden distinguirse **formas estacionarias** y **formas progresivas,** estas últimas con pérdida de visión periférica progresiva hacia la región central y posible afectación de conos en estadíos tardíos. Ejemplos de este grupo son la Retinosis pigmentaria (RP), las Distrofias de Bastones-Conos (DBC)**, la** *LCA*, etc.

El 20-30% de las DR pueden presentar afectación extra-ocular, con la implicación de distintos órganos como el oído, el riñón, el sistema endocrino, sistema cognitivo, etc. Existen más de 40 tipos de DR sindrómicas de las cuales destacan, por su prevalencia, el síndrome de Usher, el síndrome de Bardet-Bield (BBS), el síndrome de Alström (ALMS), el síndrome de SeniorLocken (SLS), la enfermedad de Norrie y el síndrome de Joubert, entre otros (Figura 5).

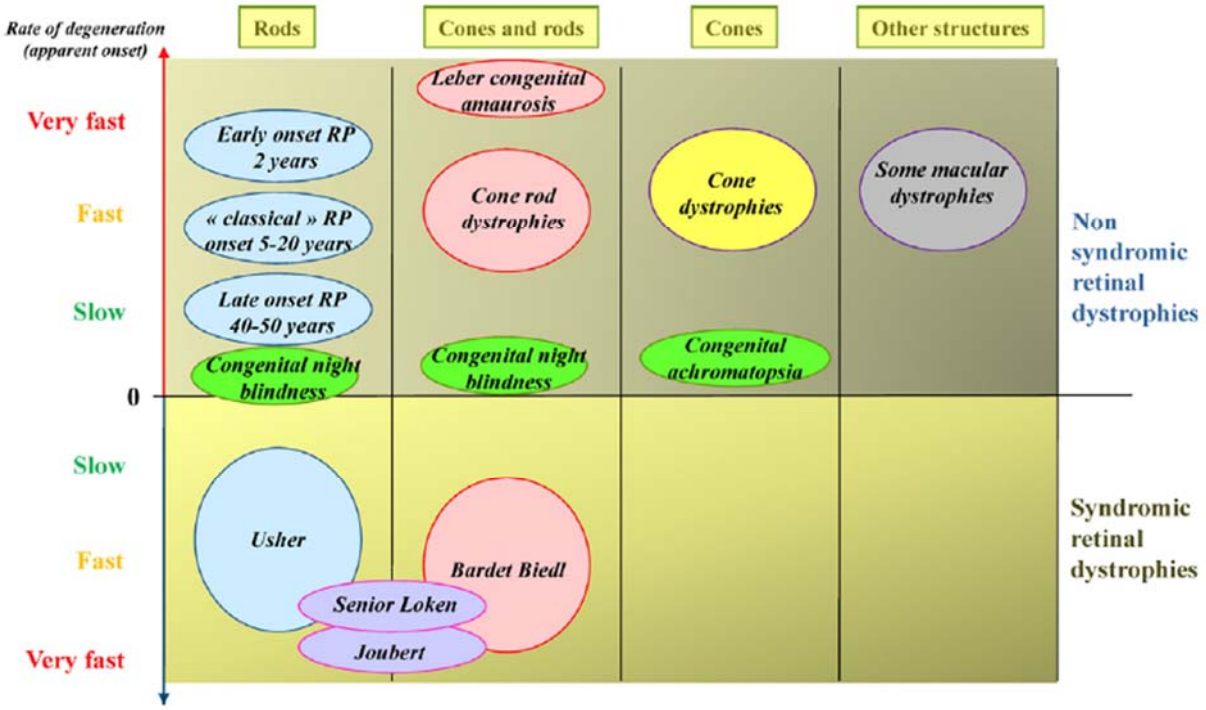

Figura 5. Clasificación de las distrofias de retina en función del tipo de receptor afectado, grado de degeneración y condición sindrómica ó no sindrómica (Hamel *et al*., 2014).

Las DR presentan una elevada heterogeneidad tanto genética, donde mutaciones en distintos genes dan lugar al mismo fenotipo, como clínica, cuando mutaciones en el mismo gen dan lugar a distintas presentaciones clínicas (Figura 6). Algunos ejemplos son el gen *PRPH2* que puede estar asociado tanto a retinosis pigmentaria como a distrofia Macular (Kohl *et al*., 2012) o el gen *ABCA4*, cuyos pacientes pueden presentar un fenotipo de enfermedad de Stargardt o distrofia de conos-bastones (Riveiro-Alvarez *et al*., 2013).

Existen más de 250 genes asociados a DR de los cuales más de 100 se asocian con hasta 40 tipos distintos de formas sindrómicas (RetNet, junio 2017). Todo ello hace que el diagnóstico genético de este grupo de patologías sea muy complejo.

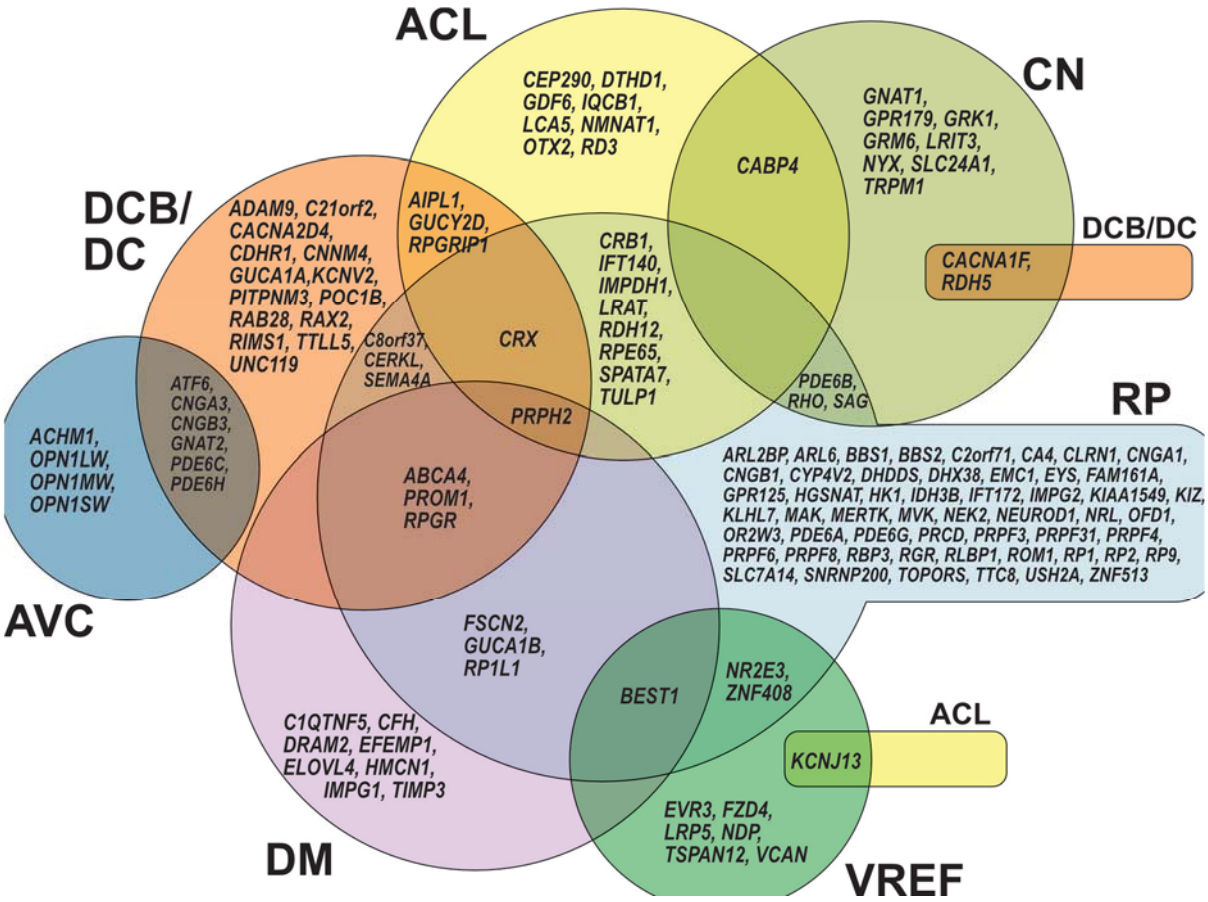

Figura 6. Diagrama de genes asociados a distrofias de retina. Las zonas solapantes indican cómo un mismo gen puede dar lugar a diferentes fenotipos. RP: retinosis pigmentaria; CN: ceguera nocturna; ACL: amaurosis congénita de Leber; DCB/DC: distrofia de conos-bastones y distrofia de conos; AVC: alteración de la visión cromática; DM: degeneración macular; VREF: vitreorretinopatía exudativa familiar. Imagen adaptada de Berger *et al.*, 2010.

3. Retinosis pigmentaria

3.1. Epidemiología

La Retinosis Pigmentaria (RP; MIM 268000) es la degeneración hereditaria más frecuente de la retina, suponiendo el 85-90% de todos los casos de DR (Ayuso & Millan, 2010). Además, constituye la primera causa de ceguera irreversible en los países desarrollados entre las personas menores de 70 años y su prevalencia es de 1:4000 individuos en Europa, estimándose en torno a 170.000 el número de individuos afectados por RP. Además, se considera que esta enfermedad provoca un impacto económico significativo y una reducción

en la calidad de vida, por lo que es importante tomar las medidas necesarias para su prevención, diagnóstico y tratamiento.

3.2. Aspectos clínicos de la RP

La RP es una distrofia primaria de bastones, que en estadíos finales cursa con degeneración secundaria de conos. Al inicio de la enfermedad, los pacientes presentan visión nocturna alterada, pérdida progresiva de la visión periférica y una alteración en la respuesta escotópica que, con el tiempo puede afectar también a los conos y por tanto a la respuesta fotópica. El fondo de ojo típico del paciente con RP presenta depósitos de pigmento en la periferia, atenuación de los capilares, papila pálida y, en algunos casos, atrofia macular. A medida que avanza la enfermedad, aparece una pérdida progresiva de la agudeza visual (AV) y del campo visual (CV), dando lugar a una visión en túnel (Marmor *et al*., 1979; Marmor *et al*., 1980; Gawande *et al*., 1989; Marmor *et al*., 1991).

En el diagnóstico oftalmológico se deben considerar los primeros síntomas de nictalopía y pérdida de visión periférica así como se debe obtener información de la edad de comienzo y progresión de la enfermedad.

Entre las pruebas oftalmológicas más relevantes para el diagnóstico de la RP destacan: i) **campimetría,** que permite la identificación de una posible disminución en el campo visual periférico, detección de islotes, escotoma central, etc; ii) **funduscopia**, que permite analizar los hallazgos oftalmológicos en el fondo de ojo típicos de este grupo de enfermedades como la presencia de depósito de pigmentos en forma de espícula, disminución del calibre de los vasos, palidez del disco óptico; iii) **electrofisiología**, que para la RP se utiliza principalmente el electrorretinograma (ERG) de campo completo que mide la respuesta eléctrica de los fotorreceptores permitiendo identificar afectación de bastones y /o conos; iv) **medida de la agudeza visual**, que proporciona información sobre el grado de afectación de los conos.

3.3. Aspectos genéticos de la RP

La RP se caracteriza por una gran complejidad a nivel genético que dificulta enormemente el estudio molecular. Se han descrito patrones de herencia autosómica dominante (adRP), autosómica recesiva (arRP), ligada al cromosoma X (xlRP), herencia digénica, bialélica y trialélica, e incluso herencia mitocondrial (Hartong *et al.,* 2006). También

aparece en formas simples o esporádicas (sRP) en familias donde hay un único miembro afectado y en las que es difícil, a priori, establecer un patrón hereditario.

Estudios epidemiológicos en población española indican que la sRP supone el 40% de las familias, seguida de las formas recesivas (38%), formas dominantes (15%) y ligadas al cromosoma X (6%). El 2% restante corresponde a formas no clasificadas. (Ayuso *et al.*, 1995). Esta distribución difiere con otras poblaciones estudiadas, donde se observa que las formas dominantes están presentes en, aproximadamente, un 30%, las formas esporádicas y no clasificadas suponen un 30%, las formas recesivas en un 25% y el 15% restante corresponde a formas ligadas al cromosoma X (Daiger *et al*., 2007). Actualmente, se han descrito hasta 80 genes asociados a RP no sindrómica (RetNet, junio 2017) y cada uno de ellos es el responsable de la enfermedad en un número reducido de familias, a excepción de algunos casos como el gen *RPGR*, presente en el 70 % de las formas ligadas al sexo (Hartong *et al.,* 2006), el gen *RHO* que representa el 20-25 % de los casos de adRP (Millá *et al.*, 2002), el gen *USH2A* que causa el 10% de las formas recesivas de RP (Blanco-Kelly *et al*., 2015) o el gen el gen *CRB1* que causa el 9% de las formas recesivas con inicio precoz (Corton *et al.,* 2013). Además, es infrecuente encontrar mutaciones prevalentes o puntos calientes, a excepción de algunos casos como la mutación p. Pro347Leu en el gen *RHO* (Ziviello *et al.*, 2005; *Audo et al*., 2010) que afecta al transporte de la rodopsina hacia el segmento externo (Rakoczy *et al*., 2011).

4. Retinosis pigmentaria autosómica dominante

Representa aproximadamente un 15% de las familias con RP en población española (Ayuso *et al.,* 1995). Con este modelo de herencia, ambos sexos tienen la misma probabilidad de verse afectados, la transmisión es vertical, los individuos tienen un 50% de probabilidad de transmitir la mutación causante de la enfermedad en cada gestación por lo que las familias presentan miembros con afectación en cada generación. Normalmente tiene un comienzo tardío y es menos severa que las formas recesivas y ligadas al cromosoma X. La mayoría de los árboles genealógicos de las familias con adRP muestran penetrancia completa, aunque también es posible encontrar familias con penetrancia incompleta, especialmente asociada a algunos genes como *PRPF31 y RP1* (Martínez-Gimeno *et al.,* 2003; Gamundi *et al.*, 2006) y expresividad variable asociadas a algunas mutaciones concretas (Kim *et al*., 1995).

La herencia autosómica dominante seevalúa de acuerdo con criterios previamente establecidos ya sea por la presencia de tres o más generaciones, con hombres y mujeres entre

todos los miembros afectados de la familia, o por lo menos dos generaciones afectadas con transmisión de varón a varón. Estos requisitos ayudan a reducir la probabilidad de incluir a las familias vinculadas al cromosoma X (Ayuso *et al.,* 1995).

4.1. Genes y proteínas implicados en adRP

Hasta la fecha existen 28 genes asociados a adRP (RetNet , junio 2017): *ADIPOR1*, *ARL3, BEST1, CA4, CRX, FSCN2, GUCA1B, HK1, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF4, PRPF6, PRPF8, PRPF31, PRPH2, RDH12, RHO, ROM1, RP1, RP9, RPE65, SEMA4A, SNRNP200, SPP2 y TOPORS*. Seis de ellos *(BEST1, NR2E3, NRL, RHO, RP1 y RPE65)* también son responsables de formas recesivas de la enfermedad. El gen *SAG,* implicado en arRP, también ha sido recientemente asociado a formas dominantes (Sullivan *et al.,* 2017).

Tabla 1: Genes asociados a adRP.

Gen	Función	Fenotipos asociados	Nº Mutaciones	Referencia
ADIPOR1	ND	adRP/arRP sd	2	(Xu *et al.*, 2016)
ARL3	Fototransducción	adRP	1	(Strom *et al.*, 2016)
BEST1	Canal iónico	adRP/adDM	237	(Petrukhin *et al.*, 1998)
CA4	ND	adRP	7	(Bardien *et al.*, 1995)
CRX	Factor de transcripción	adRP/adLCA/adDCB	53	(Freund *et al.,* 1997)
FSCN2	Estructural	adRP/adDM	1	(Bardien-Kruger *et al*., 1999)
GUCA1B	Fototransducción	adRP/adDM	3	(Payne *et al.*, 1999)
HK1	ND	adRP/arRP	5	(Sullivan *et al.,* 2014)
IMPDH1	Regulación del crecim.celular	adRP/adLCA	13	(Bowne *et al.,* 2002)
KLHL7	Ubiquitinación	adRP	3	(Friedman *et al*., 2009)
NR2E3	Factor de transcripción	adRP/arRP	50	(Kaplan et al., 1999)
NRL	Factor de transcripción	adRP/arRP	14	(Bessant *et al.,* 1999)
PRPF3	*Splicing*	adRP	3	(Chakarova *et al.,* 2002)
PRPF4	*Splicing*	adRP	4	(Chen *et al.,*2014)
PRPF6	*Splicing*	adRP	2	(Tanackovic *et al.*, 2011)
PRPF8	*Splicing*	adRP	21	(Greenberg *et al.,*1994)
PRPF31	*Splicing*	adRP	66	(Vithana *et al.,* 2001)
PRPH2	Estructural	adRP	122	(Farrar *et al.* 1991)
RDH12	Fototransducción	adRP/arLCA	66	(Haeseleer *et al.,*2002)
RHO	Fototransducción	adRP/arRP/CSNB	162	(Dryja *et al.,* 1990)
ROM1	Estructural	adRP	10	(Kajiwara et al., 1994)
RP1	Ciliar	adRP/arRP	65	(Pierce *et al.,* 1999)
RP9	*Splicing*	adRP	2	(Keen *et al.,* 2002)
RPE65	Ciclo celular	adRP/arRP/arLCA	134	(Marlhens *et al.,* 1997)
SEMA4A	Mantenimiento tisular	adRP/adDCB	3	(Kumanogoh *et al.,* 2002)
SNRNP200	*Splicing*	adRP	7	(Zhao *et al.,* 2006)
SPP2	ND	adRP	1	(Liu *et al.,* 2015)
TOPORS	Ubiquitinación	adRP	9	(Papaioannou *et al.,* 2005)

Leyenda: ND: No definido; ad: autosómico dominante; ar: autosómico recesivo; DCB: Distrofia de Conos-Bastones; DM: Distrofia Macular; LCA: *Leber Congenital Amaurosis*; RP: Retinosis Pigmentaria.

Los genes responsables de las DR pueden expresarse tanto ubicuamente, como sucede con genes implicados en el *splicing* (*PRPF3*, *PRPF8 y PRPF31*) como únicamente en retina (*RHO*). Las proteínas codificadas pueden estar implicadas en la cascada de fototransducción (*GUCA1B*, RDH12, RHO), pueden ser proteínas de membrana (*PRPH2*), factores de transcripción que regulan la expresión génica (*CRX*, *NR2E3*), cilio conector (*RP1*), ciclo visual (*RPE65*, *RDH12*), ubiquitinación (*TOPORS*), entre otras funciones. Por último, en algunos casos no se conoce la función de genes asociados a DR como sucede con *ADIPOR1 o SPP2* (Tabla 1). A continuación se describen con más profundidad los genes que se presentan con mayor frecuencia en adRP.

4.1.1. *RHO* (*Rhodopsin*)

Localizado en la posición cromosómica 3q22.1, el gen *RHO* fue el primero asociado a RP(*Dryja et al., 1990)* . Este gen codifica para la rodopsina, proteína de 348 aminoácidos que se localiza en los discos externos de los bastones, formando parte del pigmento visual de los bastones. El gen consta de cinco exones. Hasta el momento se han descrito más de 162 mutaciones asociadas a RP recopiladas en la base de datos HGMD donde el 78% corresponde a variantes *missense*, el 8% *frameshift*, el 5% *in-frame*, el 4.5% *nonsense*, el 3% de *splicing*, el 1% *stop loss* y el 0.5% corresponden a *CNVs*. La mayor parte de los cambios de una única base (*Single Nucleotide Variants, SNVs*) se distribuyen preferencialmente en los exones 1 y 3, donde se localizan más del 50% de las mutaciones. Además, se han descrito puntos calientes en el exón 1 y en la región carboxi-terminal. La mayoría de las mutaciones están involucradas en formas de RP de herencia autosómica dominante pero también han sido descritas formas recesivas (Rosenfeld *et al.,* 1992; Kartasasmita *et al.,* 2011) y casos de ceguera nocturna estacionaria de herencia autosómica dominante (Dryja *et al.,* 1993).

Actualmente el mecanismo exacto por el que mutaciones en *RHO* producen la degeneración observada en pacientes con ADRP no está del todo esclarecida. Según el efecto observado *in vitro*, las mutaciones se clasifican en 6 clases (Mendes *et al.,*2005). Las variantes de **Clase I** se localizan en el extremo carboxi-terminal. A este grupo pertenece p. Pro347Leu y se comportan de forma similar a la proteína normal en su capacidad de plagamiento, unión al 11-*cis* retinal y activación la transducina, pero no pueden ser transportados al segmento externo. Los mutantes de **Clase II**, que representan el 85% de las mutaciones en *RHO*, producen un plegamiento inadecuado de la proteína produciendo una disminución su capacidad para unirse al 11 *cis*-retinal y quedando retenidos en el RE provocando la apoptosis.

Las mutaciones de **Clase III** forman complejos rodopsina-arrestina estables que obstaculizan la endocitosis. Las variantes de **Clase IV** se localizan a nivel del extremo N-terminal ,no afectan directamente al plegamiento, pero pueden afectar a la estabilidad de la rodopsina y sus modificaciones post-traduccionales. Las mutaciones de **Clase V** provocan la activación la transducina y, por último, los mutantes de **Clase VI** afectan al residuo responsable de la unión entre la rodposina y el 11 *cis*-retinal y provocan la activación constitutiva de la transducina en ausencia del cromóforo durante la fase oscura. También se ha realizado una clasificación de las mutaciones en el gen *RHO* en función del fenotipo (Cideciyan *et al.,*1998), dividiéndose en **Clase A** los pacientes que presentan una alteración severa y generalizada de los bastones en edad temprana y progresivamente desarrollarán una alteración de los conos y en **Clase B** a los pacientes con un fenotipo más leve, incluso la alteración puede estar limitada a algunas regiones de la retina o ausente.

4.1.2. *PRPH2* (*Pheripherin 2*)

El gen *PRPH2*, también conocido como *RDS*, se encuentra localizado en la posición cromosómica 6p21.2, codifica para la proteína periferina, una glicoproteína de 347 aminoácidos que se encuentra en los discos de los fotorreceptores y tiene un papel de molécula de adhesión estabilizando y compartimentalizando el segmento externo. Con una prevalencia de un 10% (Manes *et al.,*2015), actualmente se han descrito más de 120 mutaciones en este gen (http://www.hgmd.cf.ac.uk/), 42 de ellas asociadas a adRP, y las restantes están involucradas en otros fenotipos como MD o DCB.

4.1.3. *PRPF31* (*Pre-mRNA Processing factor31*)

Localizado en la posición cromosómica 19q13.42, se compone de 14 exones que codifican para una proteína ubicua de 499 aminoácidos que juega un importante papel en la escisión de intrones durante el proceso de *splicing*. Con una prevalencia de un 7% en población española (Martin-Merida *et al.,*2017), se han descrito 66 mutaciones para este gen y todas ellas asociadas a adRP (http://www.hgmd.cf.ac.uk/).

4.1.4. *RP1* (*Retinitis Pigmentosa1 ,autosomal dominant*)

Localizado en la región pericéntrica del cromosoma 8, *RP1*es un gen que se expresa específicamente en los fotorreceptores. Está formado por 4 exones que codifican para una proteína de 2156 aminoácidos que parece estar implicada en la estabilización de la arquitectura de los discos de los segmentos externos.

Este gen ha sido relacionado tanto con formas recesivas como dominantes de RP. Para estas últimas existe un *cluster* de 442 nucleótidos en el exón 4 del gen en el que se concentran todas las variantes asociadas a adRP (Bowne *et al.,* 1999), con una prevalencia del 3,5-7 % (Bowne *et al.,* 1999; Millá *et al.,*2002).

4.1.5. *NR2E3* (*Nuclear Receptor Subfamily 2, Group E, Member 3*)

El gen *NR2E3*, localizado en 15q23, contiene 8 exones y codifica para un receptor nuclear de la retina que actúa como regulador transcripcional y está implicado en la diferenciación de los bastones. La mayoría de las mutaciones en este gen se han asociado a arRP con fenotipos variables, a excepción de la mutación, p.Gly56Arg, implicada en formas dominantes con una prevalencia de un 3,5% en familias de origen europeo (Coppieters *et al.,*2007; Blanco-Kelly *et al.,*2016).

5. Estrategias para el diagnóstico molecular de adRP

La secuenciación Sanger ha sido tradicionalmente la técnica utilizada para llevar a cabo el estudio de la adRP priorizando de manera secuencial aquellos genes con mayor prevalencia. La combinación de técnicas como *SSCP* (*Single Stranded Conformational Polymorphism*) o *DGGE* (*Denaturin Gradient Gel Electrophoresis*) junto con la secuenciación Sanger, ha facilitado este trabajo pero la elevada heterogeneidad clínica, genética y alélica existente hace que el diagnóstico molecular de la enfermedad tenga un elevado coste de tiempo y dinero.

Una alternativa ampliamente utilizada en los últimos años ha consistido en la aplicación de *arrays* de genotipado basados en la tecnología *APEX* (*Arrayed Primer Extension*) que permiten el estudio de variantes patogénicas previamente identificadas en genes conocidos. El array específico para adRP es capaz de detectar, en su última versión, hasta 414 mutaciones en 16 genes asociados a adRP (*CA4, CRX, FSCN2 KLHL7, IMPDH1, NR2E3, NRL, PRPF3, PRPF31, PRPF8, RDS, RHO, ROM1, RP1, RP9* y *TOPORS*) y con esta técnica se obtenía una tasa diagnóstica del 15% cuando se aplicó al estudio una cohorte de 139 familias (Blanco-Kelly *et al.,* 2012). Este tipo de *microarrays* ha sido evaluado en otras cohortes de nuestro grupo obteniéndose una tasa de detección de un 11% para arRP (Avila-Fernandez *et al.,* 2010), 24% para pacientes con LCA (Vallespín *et al.,* 2007) o 78% para la enfermedad de Stargardt (Aguirre-Lamban *et al.,* 2009) por lo que esta herramienta parece haber sido una buena elección como primer paso en el diagnóstico de las distrofias de retina, ya que proporciona rapidez y abarata el estudio.

El diagnóstico molecular de la adRP ha ido evolucionando a lo largo de los años, desarrollándose nuevas tecnologías que han permitido identificación de la causa de la enfermedad mediante nuevos métodos de análisis genético directo. Existen diferentes tipos de plataformas de secuenciación masiva (*NGS*) que con el tiempo han ido reduciendo su tamaño, coste y complejidad de protocolos y análisis de datos para poder implementarse en la práctica clínica en el caso de enfermedades altamente heterogéneas. La secuenciación de regiones concretas del ADN se lleva a cabo mediante amplicones o captura y marcaje con sondas. Todos ellos se basan en la secuenciación masiva de moléculas de ADN amplificadas obteniéndose cientos de millones de bases, a un reducido coste por nucleótido y por tiempo. De entre las posibles aplicaciones de la secuenciación masiva en el campo de la genómica destacan la secuenciación dirigida de determinadas regiones de interés (*targeted sequencing*), la secuenciación de exoma completo (*Whole Exome Sequencing, WES*) en la que se secuencian todos los exones codificantes y regiones flanqueantes del genoma, alrededor del 1% del genoma y la secuenciación de genoma completo (*Whole Genome Sequencing; WGS*).

Uno de los principales problemas que presenta la *NGS* es el complejo procesamiento que requiere como el alineamiento, ensamblaje, anotación e interpretación de variantes para poder transferir los datos obtenidos al diagnóstico del paciente. Además, pueden presentarse errores de lectura por un mal alineamiento de las secuencias y errores en las regiones con homopolímeros, en algunos casos, dando origen a falsos positivos y negativos. Esto hace que hoy en día se siga considerando imprescindible la comprobación de las variantes identificadas mediante otras técnicas como son la secuenciación Sanger y para la validación de *CNVs* (*Copy Number Variations*), *MLPA* (*Multiplex Ligation Probe Amplification*) o *arrays de* CGH (*Comparative Genomic Hybridization array*). Aún así, los avances en *NGS* están permitiendo su aplicación en enfermedades genéticas con elevada heterogeneidad, como son las DR, siendo una herramienta eficaz tanto para el diagnóstico de los pacientes como para la identificación de nuevos genes (Bowne *et al.,* 2011; Corton *et al.,* 2013; Perez-Carro *et al.,* 2016).

6. Perspectivas terapéuticas:

El ojo es un órgano accesible y tiene un privilegiado sistema inmune, característico debido a que sus barreras internas limitan la difusión sistémica de sustancias al resto del organismo, todo ello hace que los avances en terapia para el tratamiento de DR sean muy prometedores. Los tipos de terapias que se están desarrollando en el campo de las DR son:

Los **implantes artificiales** se basan en la estimulación eléctrica de la red neuronal de la retina que permanezca funcional, mediante la implantación de electrodos. Las limitaciones principales de estos implantes son su biocompatibilidad, estabilidad a largo plazo y la mejora de la resolución espacial (Zrenner *et al.,* 2011).

La **optogenética** está basada en la restauración de fotosensibilidad de determinadas células que todavía permanecen en la retina mediante el uso de fotosensores artificiales que permiten cambiar su potencial de membrana. Hasta ahora se trata de ensayos preclínicos en modelos de ratón con retinosis pigmentaria y retinas humanas *ex vivo* (Busskamp *et al.,* 2012).

La **terapia farmacológica** con sustancias para intentar reducir el estrés oxidativo y los procesos inflamatorios que provoca la apoptosis de los receptores. Se han llevado a cabo ensayos con vitamina A, ácidos tipo omega-3 (ácido docosahexaenoico, DHA), safranina, ácido valproico, derivados de las prostaglandinas o análogos de los retinoides. También se está valorando el efecto de los factores neurotróficos en ensayos clínicos, como el CNTF (*Cilliary Neurotrophic Factor*) que se libera al interior del globo ocular a través de una tecnología de células encapsuladas (Tao *et al.,* 2006).

Existen varios ensayos clínicos en fase I de terapia **celular** para generar información preliminar sobre el efecto y la seguridad del uso de células madre pluripotentes inducidas, células progenitoras de retina y células embrionarias que serán transplantadas en sujetos afectados de distrofias de retina.

Por último, la **terapia génica** actualmente es una de las estrategias más prometedoras. Existen ensayos clínicos en pacientes con Amaurosis Congénita de Leber producida por defectos en el gen *RPE65* mediante la inyección subretiniana de vectores AAV (*Adeno-Associated Virus*) con el gen funcional (Barker *et al.,* 2009). También existen ensayos en pacientes afectados de coroideremia con mutaciones en el gen *CHM* (MacLaren *et al.,* 2014). Para el gen *RHO*, se ha utilizado con éxito la inyección de ARN de interferencia (Mao *et al.,* 2012) y otros represores transcripcionales (Kabadi & Gersbach, 2014) para tratar de silenciar el gen en varios modelos animales. En la actualidad, con el avance en técnicas de edición génica como el sistema *CRISPR* (*Clustered Regularly Interspaced Short Palindromic Repeats*) asociado a distintos tipos de proteínas nucleasas están siendo utilizadas para la edición de genes, agregando, interrumpiendo o cambiando las secuencias de genes específicos y para la regulación génica lo que abre perspectivas muy prometedoras en el campo de las DR.

CAPÍTULO II

Objetivos

Objetivos

Objetivo principal

El objetivo principal de esta tesis ha sido ampliar el conocimiento sobre las bases genéticas y moleculares de la Retinosis Pigmentaria autosómica dominante (adRP), identificando mutaciones y genes responsables mediante la aplicación de distintas técnicas convencionales y de secuenciación masiva y su traslación a la práctica clínica, optimizando el diagnóstico genético de esta enfermedad y estableciendo correlaciones genotipo-fenotipo .

Objetivos específicos

1. Caracterizar clínica y genéticamente familias afectadas de adRP aplicando técnicas moleculares convencionales y de nueva generación.

2. Establecer la implicación de las mutaciones en el gen *RHO* en una cohorte de pacientes españoles con sospecha de adRP.

3. Desarrollar y validar una estrategia de secuenciación masiva (*Next Generation Sequencing, NGS*) basada en un panel de genes asociados a distrofias de retina para detectar las causas moleculares asociadas a adRP.

4. Aplicar secuenciación exómica completa (*Whole Exome Sequencing, WES*) a pacientes afectados de adRP con el fin de identificar nuevas mutaciones y genes implicados en la enfermedad.

5. Elaborar un algoritmo que permita un diagnóstico molecular rápido y eficiente de los pacientes afectados de adRP.

6. Establecer correlaciones genotipo-fenotipo.

CAPÍTULO III

Implicación del gen *RHO* en ADRP: cribado mutacional, correlación genotipo-fenotipo y propuesta de algoritmo diagnóstico

Artículo: *Prevalence of Rhodopsin mutations in Autosomal Dominant Retinitis Pigmentosa in Spain: clinical and analyticalreview in 200 families.*

En este trabajo se analizaron los datos clínicos y genéticos de una amplia cohorte de 200 familias diagnosticadas de adRP recogidas a lo largo de 23 años de estudio y se realizó un análisis centrado en los resultados relativos al gen *RHO*, el responsable en mayor proporción de los casos de RP a nivel mundial. Para ello, se utilizaron diferentes herramientas de detección molecular en función de su disponibilidad en nuestro laboratorio incluyendo: i) SSCP o DGGE y posterior secuenciación de las muestras con patrón alterado, ii) un microarray de genotipado específico para adRP y iii) el cribado directo mediante secuenciación Sanger de los 5 exones y regiones flanqueantes del gen *RHO*.

Un total de 42 familias presentaron mutaciones en *RHO* dando como resultado una prevalencia de un 21 % en nuestra cohorte. De esta forma se identificaron un total de 27 mutaciones distintas, de las cuales, un tercio no habían sido descritas hasta la fecha. Estas últimas fueron clasificadas como probablemente causantes de la enfermedad basándose en las características clínicas que presentaban los pacientes, su cosegregación con la patología en la familia, su consecuencia patogénica, la conservación evolutiva de los aminoácidos afectados, la predicción de patogenicidad en estudios *in silico* y por último, su ausencia en más de 200 cromosomas de población control española.

Por otro lado, se ha podido establecer una correlación genotipo-fenotipo para la mutación más frecuente, detectada en el 4,5% del total de nuestra cohorte, p.Pro347Leu, utilizando los datos oftalmológicos de 107 pacientes de las 42 familias afectadas de RP por mutaciones en *RHO*. Así se ha podido asociar este cambio a un fenotipo característico de inicio más temprano y curso más severo en comparación con otras variantes detectadas en dicho gen, permitiéndonos dar un pronóstico más adecuado a los pacientes y familiares que presentan esta mutación.

Por último, se ha propuesto un algoritmo diagnóstico coste-efectivo, teniendo en cuenta la tecnología aplicada en las familias en el momento de realización de este trabajo, concluyendo que el array de genotipado, que cubre 414 mutaciones en 16 genes distintos asociados a adRP, seguido de la secuenciación directa de los 5 exones del gen *RHO* son los

primeros pasos propuestos para el abordaje del diagnóstico molecular de familias españolas con adRP.

Este estudio retrospectivo ha permitido determinar la implicación real del gen *RHO* en nuestra cohorte y además, se ha podido detectar la causa genética de la enfermedad en un número elevado de pacientes, lo que les ha proporcionado un diagnóstico, pronóstico y consejo genético y reproductivo apropiados. Por otra parte estos pacientes, por haber sido identificada la causa genética de su enfermedad, podrán ser seleccionados como candidatos para su participación futura en ensayos de terapia farmacológica y gen-dependiente.

Aportación de la autora

En este trabajo, la autora de la memoria recopiló la información clínica y genética de las 200 familias con diagnóstico de adRP del Servicio de Genética, secuenció el gen *RHO* en 17 casos índice del total de las 200 familias, los cuales no habían sido caracterizados tras el análisis por *microarray* durante el tiempo en el que se desarrolló este estudio y segregó los cambios detectados en este gen en 4 familias. Además, la doctoranda realizó una revisión de las mutaciones publicadas previamente en bases de datos y en la literatura y la evaluación de la patogenicidad de las nuevas variantes encontradas en este estudio. Para ello, realizó los estudios de conservación de aminoácidos para mutaciones *missense* utilizando 24 ortólogos de la proteína rodopsina pertenecientes a diferentes ramas evolutivas y aplicó distintas herramientas de predicción de patogenicidad. También realizó el análisis estadístico de los resultados obtenidos con cada una de las distintas tecnologías que fueron utilizadas y colaboró en la propuesta del algoritmo diagnóstico basada en los resultados de este trabajo. Además, recopiló la información oftalmológica tras revisión de la historia clínica de 107 pacientes portadores de mutaciones en *RHO* realizandolos análisis estadísticos para establecer las correlaciones genotipo-fenotipo. Por último, la doctoranda preparó un manuscrito para la publicación del trabajo que se presenta a continuación.

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Prevalence of *Rhodopsin* mutations in autosomal dominant Retinitis Pigmentosa in Spain: clinical and analytical review in 200 families

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ABSTRACT.

Purpose: **We aimed to determine the prevalence of mutations in the *RHO* gene in Spanish families with autosomal dominant Retinitis Pigmentosa (adRP), to assess genotype–phenotype correlations and to establish an accurate diagnostic algorithm after 23 years of data collection.**

Patients and Methods: **Two hundred patients were analysed through a combination of denaturing gradient gel electrophoresis, single-strand conformation polymorphism, genotyping microarray and Sanger sequencing of the *RHO* gene.**

Results: **Overall, 42 of 200 Spanish adRP families were mutated for *RHO* (21.0%). Twenty-seven different *RHO* mutations were detected; seven of them were novel. A genotype–phenotype correlation was established with clinical data from 107 patients. The most prevalent p.Pro347Leu mutation, responsible for 4.5% (9/200) of all mutated adRP families, was associated with a phenotype of early onset and severe course diffuse RP.**

Conclusions: **This retrospective study provides a wide spectrum of mutations in the *RHO* gene in Spanish patients with adRP. Also, the prevalence of mutations is similar to that reported in European population. Genotyping microarray followed by *RHO* sequencing is proposed as a first step in molecular diagnosis of adRP Spanish families. An increasing understanding of causal *RHO* alleles in adRP facilitates disease diagnosis and prognosis, especially for the prevalent p.Pro347Leu mutation.**

Key words: autosomal dominant – diagnostic algorithm – genotype–phenotype correlation – Retinitis Pigmentosa – *RHO* – Spanish families

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Introduction

Retinitis Pigmentosa (RP) is the most common form of inherited retinopathy, with a prevalence of approximately 1:4000 (Hamel 2006). It is a group of inherited retinal diseases that progressively affect photoreceptors, thus altering their function and leading to progressive and irreversible vision loss (Hartong et al. 2006). Clinical symptoms include night blindness, reduced peripheral vision, decreased visual acuity, abnormal retinal electrophysiology and characteristic changes in the ocular fundus.

Genetic inheritance heterogeneity of this disorder includes autosomal dominant, autosomal recessive and X-linked transmission; digenic and mitochondrial forms of RP have also been described. Up to date, 56 genes have been associated with non-syndromic RP (http://www.sph.uth.tmc.edu/retnet).

Autosomal dominant Retinitis Pigmentosa (adRP) accounts for approximately 15% of Spanish RP families (Ayuso et al. 1995). The large number of genes involved in adRP disease complicates the genetic analysis of these patients. Presently, 24 genes have been mapped and identified as being associated with adRP (http://www.sph.uth.tmc.edu/retnet): *BEST1*, *CA4*, *CRX*, *FSCN2*, *GUCA1B*, *IMPDH1*, *KLHL7*, *NR2E3*, *NRL*, *PRPF3*, *PRPF4*, *PRPF6*, *PRPF8*, *PRPF31*, *PRPH2*, *RDH12*, *RHO*, *ROM1*, *RP1*, *RP9*, *RPE65*, *SEMA4A*, *SNRNP200* and *TOPORS*.

Mutations in the gene-encoding rhodopsin, the visual pigment that initiates

the phototransduction cascade in rod photoreceptors (Nathans et al. 1986). (*RHO*; OMIM+180380), are the most common cause of adRP (16% to 26%) (Ayuso et al. 1995; Millá et al. 2002; Ziviello et al. 2005; Sullivan et al. 2006). Currently, there are over 120 mutations identified in the *RHO* gene causing adRP, autosomal dominant congenital stationary night blindness (adCSNB) and autosomal recessive Retinitis Pigmentosa (arRP) (http://www.sph.uth.tmc.edu/retnet).

The most prevalent mutation in the *RHO* gene observed in European cohort of patients with adRP is p.Pro347Leu, which affects the transport of the rhodopsin protein to the outer segment (Rakoczy et al. 2011).

The present study aimed (1) to determine the prevalence of mutations in the *RHO* gene in families with adRP from Spain, (2) to assess genotype–phenotype correlations and (3) to establish an accurate diagnostic algorithm for these families.

Patients and Methods

Recruitment of subjects

A subset of 200 adRP-unrelated families were recruited at the Fundacion Jimenez Diaz University Hospital from 1990 to 2013. This study was reviewed and approved by the Ethics Committee of the Hospital, and it was performed according to the tenets of the Declaration of Helsinki and further reviews (59th WMA General Assembly, Seoul, Korea, October 2008). The participating subjects, or their legal guardians, signed a written informed consent form after the nature of procedures had been explained fully. The collection of samples belongs to the Biobank of the Fundacion Jimenez Diaz University Hospital.

Molecular methods

Genomic DNA was extracted from peripheral blood leucocytes using salting-out method (Maniatis et al. 1982) or automated DNA extractors: BioRobot EZ1 (*Qiagen*, Hilden, Germany) and MagNA Pure Compact system (*Roche Applied Science*, Penzberg, Germany).

A cohort of 200 patients with adRP was tested for variants on the *RHO* gene using a combined strategy of genotyping tools. First, 34 patients were screened by single-strand conformation polymorphism (SSCP), and 84 were analysed by CG-clamped denaturing gradient gel electrophoresis (DGGE). Those patients showing an abnormal electrophoretic migration pattern were further analysed by sequencing (Reig et al. 1994; Trujillo et al. 1998). Next, 66 uncharacterized patients, together with 101 not previously screened patients with adRP (Blanco-Kelly et al. 2012), were screened by the AD-RP genotyping microarray (*Asper Biotech*, Tartu, Estonia) (Kurg et al. 2000); all identified variants were confirmed by direct sequencing using PCR primers described previously (Nathans et al. 1986). Finally, 86 patients were selected and subsequently screened for mutations in the *RHO* gene by direct sequencing.

Assessment of the pathogenicity of new variants

The pathogenicity of unreported variants was established according to the following criteria: (1) cosegregation within the family, (2) absence in 100 Spanish healthy control individuals, (3) assessment of amino acid conservation for missense mutations using 24 orthologs of the rhodopsin protein belonging to different evolutionary branches and (4) pathogenicity prediction with *in silico* tools, as previously described (Corton et al. 2013).

Clinical evaluation

Diagnosis of adRP was determined according to a dominant mode of inheritance in patients with night blindness, peripheral vision loss, pigmentary retinal degeneration and reduced scotopic response.

The clinical ophthalmic examination included the assessment of visual acuity, intra-ocular pressure, ocular motility, pupillary reaction, biomicroscopic slit-lamp examination and dilated fundus examination. Visual function was performed by static perimetry, D15 panel testing and Ganzfeld electroretinography according to the International Society for Clinical Electrophysiology of Vision (ISCEV) Standards (Marmor & Zrenner 1993, 1999) with a UTAS 2000 system (LKC Technologies, Gaithersburg, MD, USA) and jet electrodes.

Visual field defects were classified into: normal; diffuse-relative scotoma (presence of general decrease in sensitivity); peripheral constriction (peripheral reduction with scotoma absolute over central 10°; the preserved central visual field was specified in degrees); annular scotoma (scotoma in the 20° to 80°); tubular field (10° central preserved); and tubular field with islands and absolute scotoma (0° preserved).

Statistical analysis

Statistical analysis was performed using the STATA/SE 10.0 software (StataCorp.2007. Stata Statistical Software: Relase 10. College Station, TX, USA).

Results

Mutation detection rate and spectrum of *Rhodopsin* mutant variants

Through the combined methodological approach, 27 different *RHO* mutations were identified in 42 of 200 unrelated adRP families, resulting in a mutation detection rate of 21.0% (Table 1 and Figure S1). All mutations detected in the *RHO* gene were found in families of Spanish origin, with the exception of the p.Trp126* mutation, which was found in a Senegalese family (RP-1584).

Overall, 27 different mutant alleles were detected; 25 of 27 (92.6%) were missense variants, 1 of 27 (3.7%) was affecting a splice site, and 1 of 27 (3.7%) was a nonsense mutation.

Additionally, seven novel changes were identified in the *RHO* gene: six were missense (p.Gln28His, p.Leu79Pro, p.Thr97Ile, p.Met163Thr, p.Cys167Tyr and p.Gly182Val), and one resulted in a stop-codon mutation (p.Trp126*). These novel *RHO* variants were predicted as pathogenic based on both the highly evolutionary conservation (Fig. 1) and pathogenicity prediction using '*in silico*' programs; cosegregation within the families (when possible) and their absence in more than 200 chromosomes of the control population (Table 2).

The p.Pro347Leu variant was the most frequent mutation in *RHO*, resulting the 33.3% (9/27) of all mutant alleles. This mutation was identified in nine unrelated families, therefore representing the 4.5% of our cohort of patients with adRP.

Table 1. Spectrum of *RHO* mutations in autosomal dominant Retinitis Pigmentosa families. Novel mutations are shown in bold. Legends: autosomal dominant Retinitis Pigmentosa genotyping microarray (AD-RP chip); denaturing gradient gel electrophoresis (DGGE); single-strand conformation polymorphism (SSCP); Sanger sequencing (SS); not performed (NP); stop codon (*). Nucleotide numbering reflects cDNA in the reference sequence NM_000539.

Family	Exon	Nucleotide change	Amino Acid change	Methods	Segregation	References
RP-1927	1	c.44A>G	p.Asn15Ser	AD-RP chip	Yes	(Kranich et al. 1993)
RP-0706	1	c.50C>T	p.Thr17Met	DGGE	NP	(Sheffield et al. 1991)
RP-2179	1	**c.84G > C**	**p.Gln28His**	AD-RP chip	NP	**This study**
RP-0041	1	c.131T>C	p.Met44Thr	DGGE	Yes	(Reig et al. 1994)
RP-0820	1	c.173C>G	p.Thr58Arg	DGGE	NP	(Dryja et al. 1990)
RP-1908	1	c.173C>G	p.Thr58Arg	AD-RP chip	NP	(Dryja et al. 1990)
ONCE-0169	1	**c.236T > C**	**p.Leu79Pro**	DGGE	NP	**This study**
RP-0890	1	**c.290C > T**	**p.Thr97Ile**	DGGE	NP	**This study**
RP-0242	1	c.316G>A	p.Gly106Arg	DGGE	Yes	(Berson et al. 1991; Ayuso et al. 1996)
RP-0515	1	c.316G>A	p.Gly106Arg	AD-RP chip	Yes	(Berson et al. 1991)
RP-1584	1	**c.378G > A**	**p.Trp126***	SS	NP	**This study**
RP-0314	2	c.403C>T	p.Arg135Trp	DGGE	Yes	(Sung et al. 1991)
RP-1188	2	c.404G>T	p.Arg135Leu	AD-RP chip	Yes	(Andréasson et al. 1992)
RP-1876	2	c.404G>T	p.Arg135Leu	AD-RP chip	NP	(Andréasson et al. 1992)
RP-0269	2	**c.488T > C**	**p.Met163Thr**	SS	NP	**This study**
RP-0727	3	c.491C>A	p.Ala164Glu	AD-RP chip	Yes	(Vaithinathan et al. 1994)
RP-1930	2	c.491C>A	p.Ala164Glu	AD-RP chip	NP	(Vaithinathan et al. 1994)
RP-1458	2	**c.500G > A**	**p.Cys167Tyr**	SS	Yes	**This study**
RP-1941	2	**c.500G > A**	**p.Cys167Tyr**	SS	NP	**This study**
RP-2021	2	c.509C>G	p.Pro170Arg	AD-RP chip	NP	(Sohocki et al. 2001)
RP-1125	2	c.512C>T	p.Pro171Leu	AD-RP chip	Yes	(Dryja et al. 1991)
RP-0568		c.531-2A>G	Splicing site	DGGE	Yes	(Martinez-Gimeno et al. 2000)
RP-0913	3	c.533A>G	p.Tyr178Cys	AD-RP chip	Yes	(Sung et al. 1991)
RP-1672	3	c.541G>A	p.Glu181Lys	SS	Yes	(Dryja et al. 1991)
RP-0640	3	c.544G>A	p.Gly182Ser	DGGE	Yes	(Sheffield et al. 1991)
RP-1375	3	c.544G>A	p.Gly182Ser	AD-RP chip	Yes	(Sheffield et al. 1991)
RP-1952	3	**c.545G > T**	**p.Gly182Val**	SS	Yes	**This study**
RP-0115	3	c.556T>C	p.Ser186Pro	SSCP	NP	(Dryja et al. 1991; Trujillo et al. 2000)
RP-0352	3	c.562G>A	p.Gly188Arg	DGGE	Yes	(Dryja et al. 1991; Reig et al. 2000)
RP-0188	3	c.568G>T	p.Asp190Tyr	DGGE	Yes	(al-Maghtheh et al. 1993; Martínez Gimeno et al. 2000)
RP-0328	3	c.568G>T	p.Asp190Tyr	AD-RP chip	Yes	(al-Maghtheh et al. 1993)
RP-0566	3	c.644C>T	p.Pro215Leu	DGGE	Yes	(Martínez-Gimeno et al. 2000)
RP-0206	4	c.865A>C	p.Thr289Pro	DGGE	Yes	(Martinez-Gimeno et al. 2000)
RP-0220	5	c.1040C>T	p.Pro347Leu	SSCP	Yes	(Dryja et al. 1990; Trujillo et al. 1998)
RP-0542	5	c. 1040C>T	p.Pro347Leu	DGGE	Yes	(Dryja et al. 1990)
RP-0685	5	c. 1040C>T	p.Pro347Leu	DGGE	NP	(Dryja et al. 1990)
RP-0900	5	c. 1040C>T	p.Pro347Leu	AD-RP chip	NP	(Dryja et al. 1990)
RP-0975	5	c. 1040C>T	p.Pro347Leu	DGGE	Yes	(Dryja et al. 1990)
RP-1453	5	c. 1040C>T	p.Pro347Leu	AD-RP chip	Yes	(Dryja et al. 1990)
RP-1472	5	c. 1040C>T	p.Pro347Leu	AD-RP chip	NP	(Dryja et al. 1990)
RP-1697	5	c. 1040C>T	p.Pro347Leu	AD-RP chip	Yes	(Dryja et al. 1990)
RP-2181	5	c. 1040C>T	p.Pro347Leu	AD-RP chip	NP	(Dryja et al. 1990)

Prevalence of *RHO* mutations in autosomal dominant Retinitis Pigmentosa families

The mutational screening using the AD-RP genotyping microarray was performed in 167 patients with adRP; of these, 139 families were previously described (Blanco-Kelly et al. 2012). Overall, we identified the causative mutation in 34 of 167 patients, resulting in a mutation detection rate of 20.4%. Disease-associated alleles were identified in the following genes: *RHO* (18/167; 10.8%), *PRPF31* (4/167; 2.4%), *PRPF3* (3/167; 1.8%), *RP1* (3/167; 1.8%), *NR2E3* (2/167; 1.2%), *PRPH2* (2/167; 1.2%), *CRX* (1/167; 0.6%) and *IMPDH1* (1/167; 0.6%). Of those, mutations in the *RHO* gene were detected in 18 patients with adRP, therefore resulting in a specific mutation detection rate for *RHO* of 10.8% (18/167).

Analytical characteristics of the screening methods for the *Rhodopsin* gene

Over 23 years, 200 adRP families were subsequently analysed for mutations in the *RHO* gene using different techniques. The respective mutation detection rates were as following: 5.9% for SSCP, 19.0% for DGGE, 10.8% for AD-RP genotyping microarray specifically for the *RHO* gene and 7.0% for Sanger sequencing. When the mutation rate is considered for the 17 adRP genes included in the genotyping microarray, the rate increases to 20.4% (Table S1).

As all identified variants were confirmed by direct sequencing, sensitivity and specificity values for the AD-RP genotyping microarray were further determined, resulting in 94% and 100%, respectively (one false negative for *RHO* p.Glu181Lys mutation).

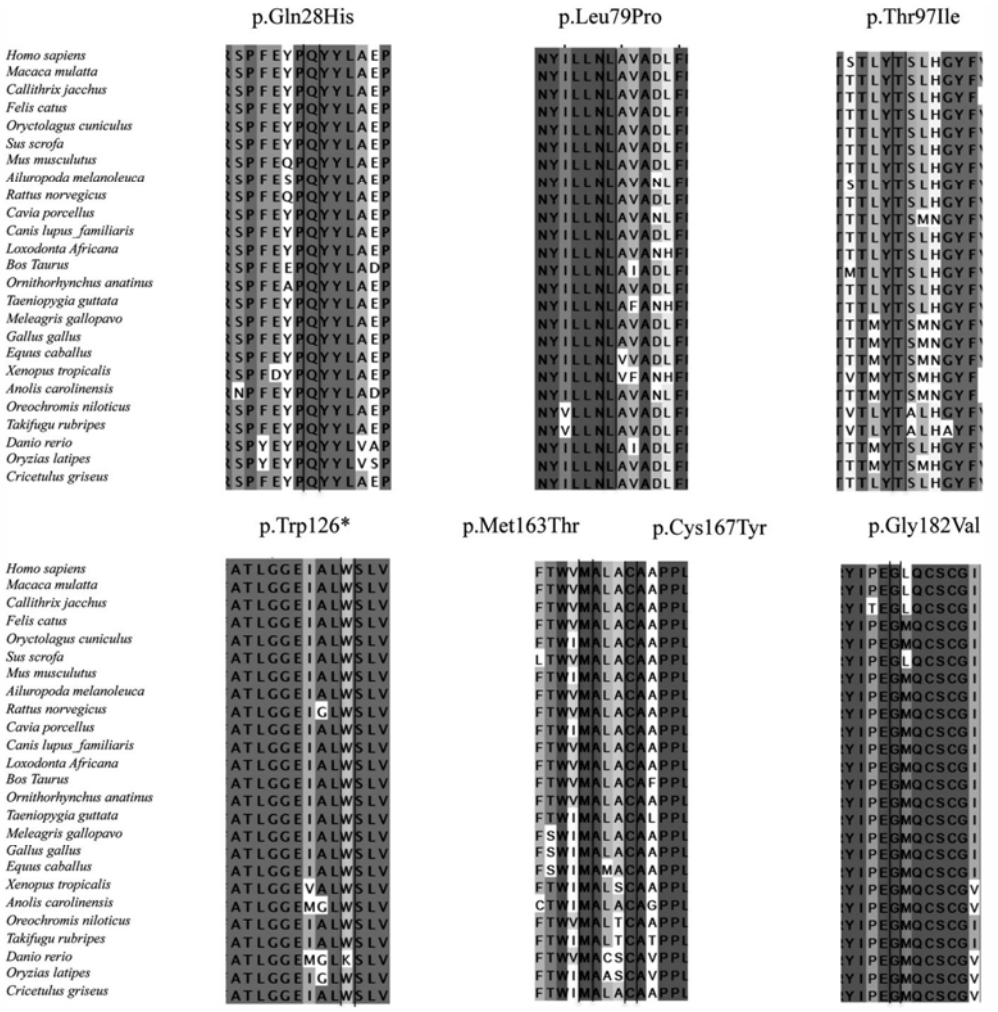

Fig. 1. Conservation of the novel amino acid substituted or deleted detected in our cohort of patients in 24 species.

Genotype–phenotype correlation

The summary of clinical and genetic findings from 107 patients belonging to the 42 *RHO*-mutated RP families is presented in Table S2.

Three main general symptoms were observed: night blindness (as the first noticeable symptom), concentric visual field reduction, followed by decreased visual acuity. Overall, electroretinogram (ERG) recordings (Fig. S2) were impaired or abolished; mainly, the scotopic signals were non-recordables at the average age of 38 years. Fundus examination showed retinal changes consisting with typical RP fundus, namely mobilizations of retinal pigment, narrow vessels and optic disc pallor. Interestingly, two patients with the p.Gly106Arg and p.Pro170Arg mutations (age at examination: 8 and 17 years, respectively) presented ERG alterations, but not fundus abnormalities.

We specially analysed the genotype–phenotype correlation between patients presenting the prevalent p.Pro347Leu mutation and patients presenting different mutations in the *RHO* gene (Table S3). Statistically significant differences were observed between both the groups, in terms of age at diagnosis ($p < 0.0001$), onset of night blindness ($p = 0.0001$), visual field reduction ($p = 0.0004$) and visual acuity loss ($p = 0.0008$).

In general, clinical data from patients with the p.Pro347Leu mutation demonstrated subtle intra- and

Table 2. List of novel *RHO* likely pathogenic variants identified in the studied cohort. Conservation of the amino acid substituted or deleted in 24 species is detailed. All novel mutations were absent in 200 control chromosomes. The amino acid substitution is predicted damaging if the SIFT score is < = 0.05. Poly-Phen-2 predicts a non-synonymous variant as benign, possibly damaging or probably damaging, if score is < 0.2, between 0.2 and 0.85 or > 0.85. GV: Grantham variation; GD: Grantham distribution. Class C65: most likely pathogenic, Class C0: less likely pathogenic. Legends: highly conserved (HC); not performed (NP); stop codon (*).

				SIFT		PolyPhen		GVGD			
Family	Nucleotide change	AA change	Conservation	Score	Prediction	Score	Prediction	GV Score	GD Score	GD-GV scores	Segregation
adRP-2179	c.84G>C	p.Gln28His	HC	0.00	Affect protein function	0.999	Probably damaging	0.00	24.08	Class C15	NP
ONCE-0169	c.236T>C	p.Leu79Pro	HC	0.00	Affect protein function	1	Probably damaging	0.00	97.78	Class C65	NP
adRP-0890	c.290C>T	p.Thr97Ile	HC	0.00	Affect protein function	0.990	Probably damaging	0.00	159.94	Class C65	NP
adRP-1584	c.378G>A	p.Trp126*	HC	–	–	–	–	–	–	–	NP
adRP-0269	c.488T>C	p.Met163Thr	HC	0.00	Affect protein function	0.993	Probably damaging	0.00	81.04	Class C65	NP
adRP-1458	c.500G>A	p.Cys167Tyr	HC	0.00	Affect protein function	0.999	Probably damaging	0.00	109.55	Class C65	YES
adRP-1941	c.500G>A	p.Cys167Tyr	HC	0.00	Affect protein function	0.999	Probably damaging	0.00	109.55	Class C65	NP
adRP-1952	c.545G>T	p.Gly182Val	HC	0.00	Affect protein function	1	Probably damaging	0.00	109.55	Class C65	YES

interfamilial variability (i.e. early onset, severe evolution and a diffuse Retinitis Pigmentosa fundus).

Discussion

As the *RHO* gene was identified (Nathans et al. 1986), mutations in this gene have been associated with two autosomal dominant retinal phenotypes (adRP and adCSNB) and autosomal recessive RP (arRP). Additionally, the role of *RHO* mutations has been analysed extensively in the Spanish population (Ayuso et al. 1995; Millá et al. 2002) and is well known. Therefore, this study was conducted to summarize and review the analytical and clinical data and to further describe the genotype–phenotype correlations between the different *RHO* disease-associated variants.

The mutation detection rate in the *RHO* gene responsible for adRP was 21%, being consistent with previously profiled data among Spanish (19.5%) (Millá et al. 2002) and higher than the frequencies found in other European populations (Italian, German and French around 16%) (Bunge et al. 1993; Ziviello et al. 2005; Audo et al. 2010).

In this work, a novel potential pathogenic variant p. Trp126* was found in the exon 2 of *RHO* (family RP-1584). Some authors consider that nonsense mutations in the *RHO* gene are recessive alleles (Rosenfeld et al. 1992; Kumaramanickavel et al. 1994; Kartasasmita et al. 2011) unless they happen in the last exon (Sung et al. 1991). Nerveless stop-codon mutations in other exons have also been associated with adRP (Macke et al. 1993; Sánchez et al. 1996; Eisenberger et al. 2013).

Through 23 years of patients recruitment, different molecular screening tools were used, namely SSCP, DGGE, adRP genotyping microarray and Sanger sequencing of the *RHO* gene. Taking into account their respective mutation detection rates (Table 2), we consider the genotyping microarray as the most appropriate technique for preliminary adRP patients screening because of: (1) the higher mutation detection rate for mutations in the *RHO* gene in comparison with SSCP/DGGE; (2) the sensitivity (94%) and specificity (100%) values; and (3) the additional mutational screening of 16 adRP-associated genes, in contrast to *RHO*-specific SSCP/DGGE techniques. The main handicap of the AD-RP chip is that it only detects the specific variants included in the array; therefore, the use of this microarray followed by *RHO* sequencing in negative cases has been proposed as a first step in molecular diagnosis of adRP Spanish families (Blanco Kelly et al. 2012).

In relation to genotype–phenotype correlation, patients with adRP with mutations in the *RHO* gene presented a typical RP phenotype. Specifically, patients with the p.Pro347Leu mutation presented a phenotype of diffuse RP with early onset and severe course of the disease, with subtle inter- and intrafamilial variability. These findings were similar to those previously described for this mutant variant in other populations (Berson et al. 1991; Oh et al. 2003).

p.Pro347Leu is the most frequent mutation in the *RHO* gene (representing both the 4.5% of the Spanish adRP cohorts and the 33.3% of the *RHO* mutant alleles). Therefore, it could be hypothesized that the high frequency of the p.Pro347Leu variant in Spain, and also in other populations (Ziviello et al. 2005; Audo et al. 2010; Fujiki et al. 1992; Greenberg et al. 1999), is due to this mutation occurred at a CG dinucleotide, which is considered a mutation hot spot of the genome (Crow 2000).

Now, with the availability of next-generation sequencing (NGS), we suggest that complete sequencing of adRP-associated gene-coding regions, including flanking intronic sequences (splice sites), would be paramount for proper molecular diagnosis in every patient with adRP (Bowne et al. 2011) and highlight further the immense genetic heterogeneity inherent in this group of disorders, both in the context of genetic counselling and in the per-

spective of retinal gene therapy (Farrar et al. 2012).

In conclusion, despite the mutational spectrum and slight differences in terms of genotype–phenotype correlation, identifying the causative mutation is helpful in confirming diagnosis and counselling, but cannot provide a prognosis, with the exception of the prevalent p.Pro347Leu allele. Additionally, this study provides further evidence of different *RHO* mutation frequency in autosomal dominant RP across populations.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Pedigrees of adRP patients with RHO mutations and co-segregation in available family members. m/+: mutation detected in heterozygosis; +/+: wild type. Filled and unfilled symbols represent affected and unaffected persons respectively. Squares indicate males, circles females. Arrows reflect the index patients.

Figure S2. Two examples of electroretinogram (ERG) recordings. The probandus of RP-0640 and RP-2021.

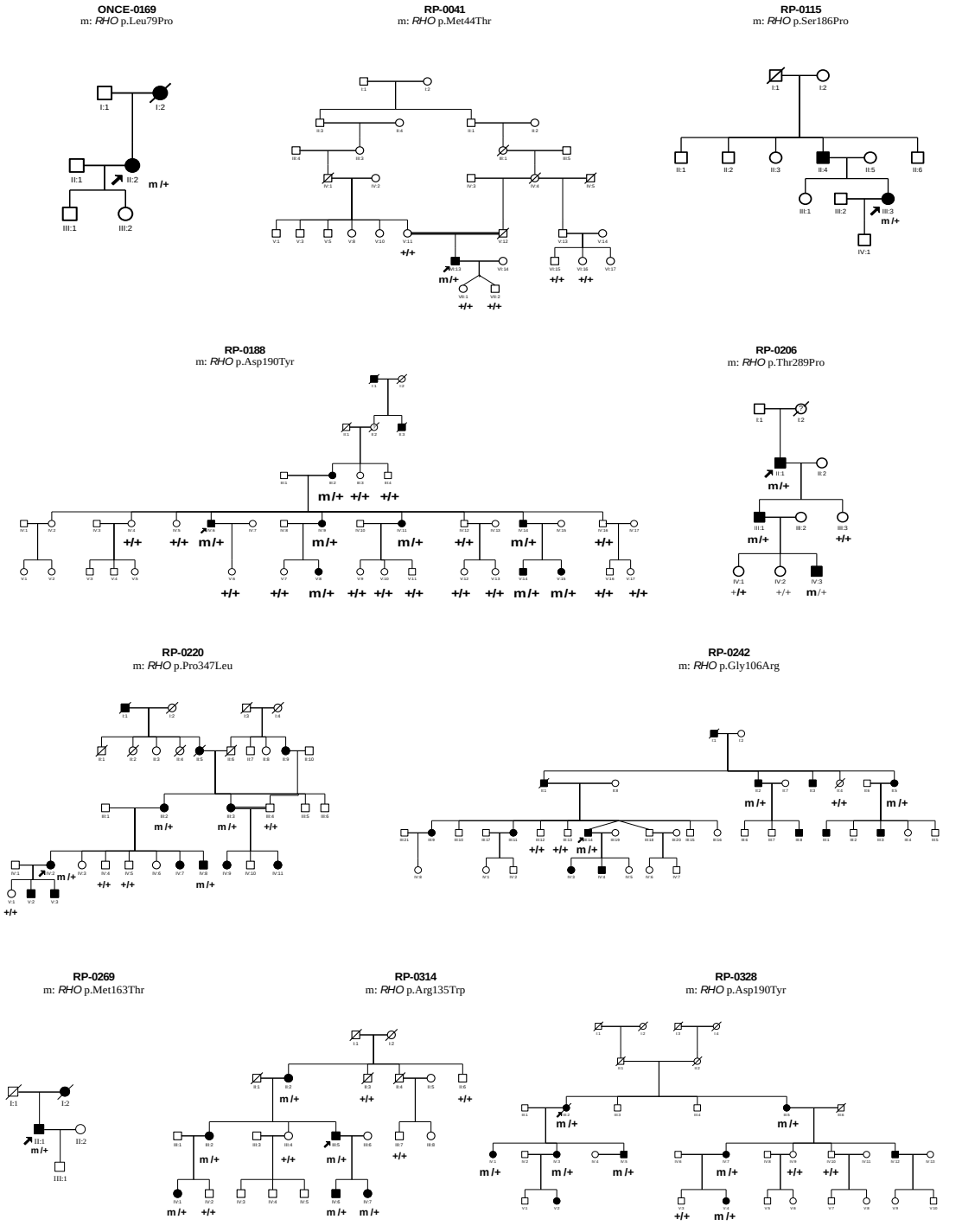
Table S1. (A) Mutation detection rates and screening methods. (B) Comparison of screening methods. The mutation detection rate increases, as expected, with the addition of other screening methods and when the results of the AD-RP microarray is considered for the 17 adRP genes, but not only for *RHO*. The number (or the fraction) of samples analysed by each method is given in parentheses.

Table S2. Clinical data of patients presenting mutation in the *Rhodopsin* gene. Legends: RP: Retinitis Pigmentosa; NA: Not Available; NR: Non Recordable; VD: Very Diminished; D: Diminished; N: Normal; VA: Visual Acuity; RE: Right Eye; LE: Left Eye.

Table S3. Age at onset of symptoms and diagnosis of all mutated patients, patients with p.Pro347Leu mutation and patients with other *RHO* mutations. *Number of patients with statistical calculations. **Comparisons were performed using the t- Student test.

Supporting information

Figure S1. Pedigrees of adRP patients with RHO mutations and co-segregation in available family members. m/+: mutation detected in heterozygosis; +/+: wild type. Filled and unfilled symbols represent affected and unaffected persons respectively. Squares indicate males, circles females. Arrows reflect the index patients.



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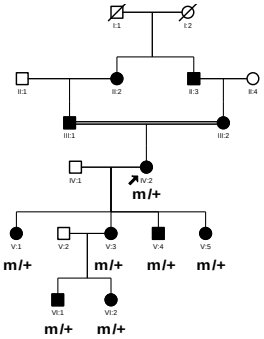
m: *RHO* p.Gly188Arg



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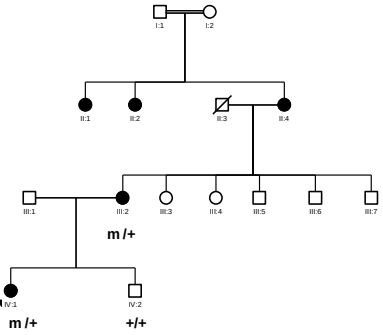
m: *RHO* p.Gly106Arg



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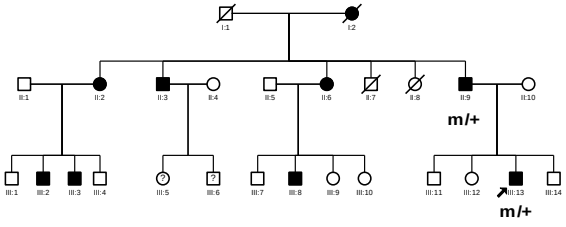
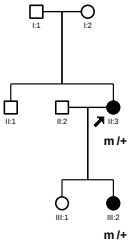
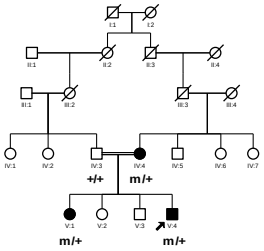
m: *RHO* p.Pro347Leu



RP-0566
m: *RHO* p.Pro215Leu



RP-0568
m: *RHO* IVS2 as A-G -2



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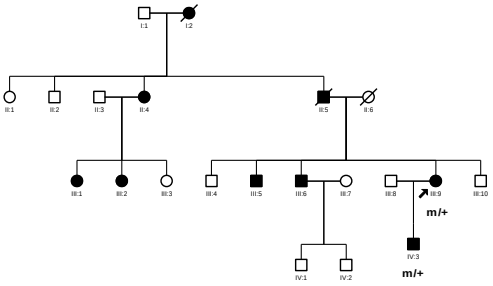
m: *RHO* p.Gly182Ser



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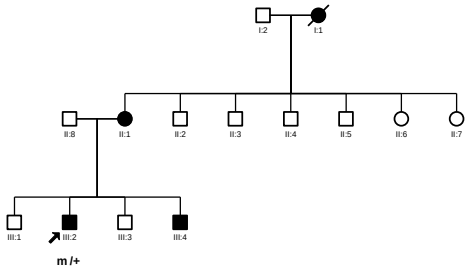

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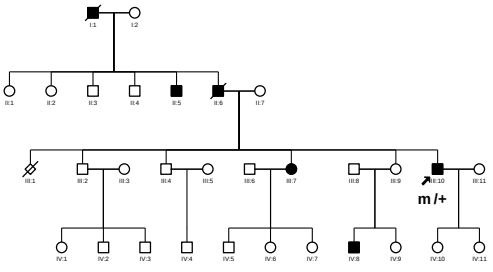

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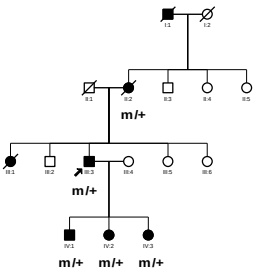

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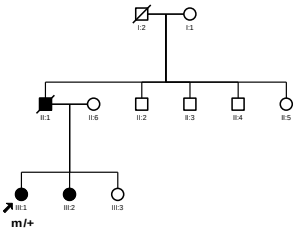
m: *RHO* p.Thr58Arg



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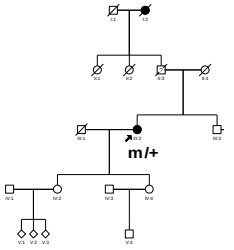
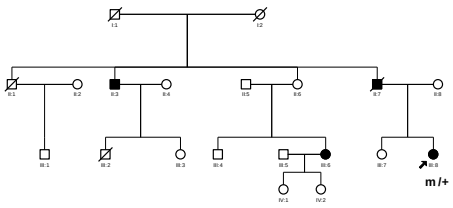


RP-0900
m: *RHO* p.Pro347Leu



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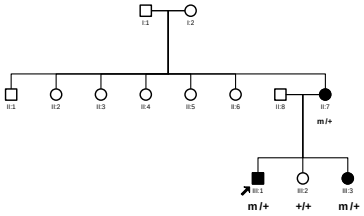

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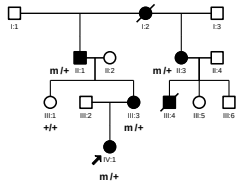

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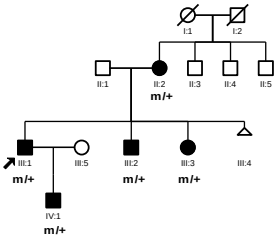

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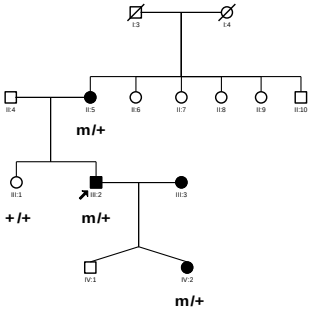

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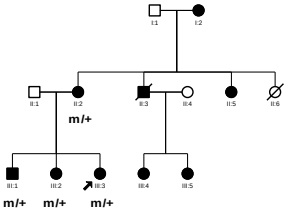

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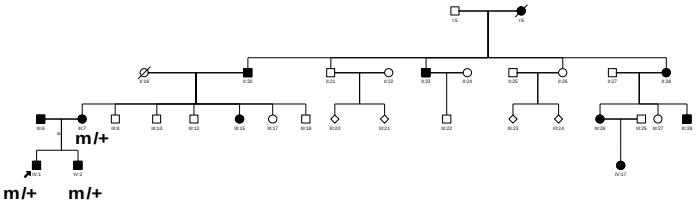

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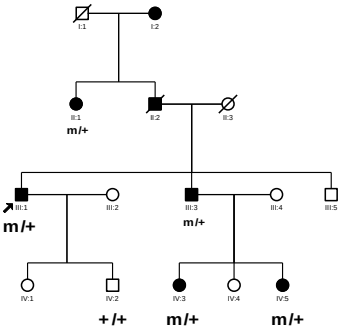

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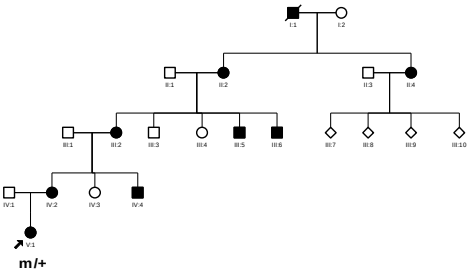

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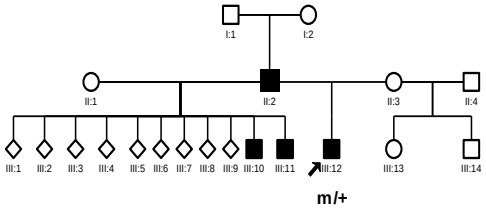

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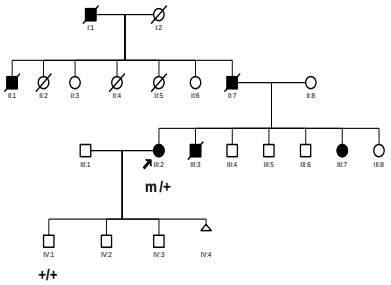

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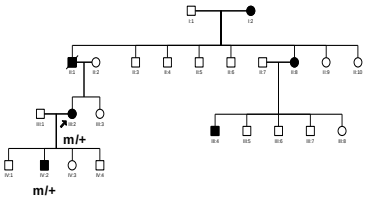

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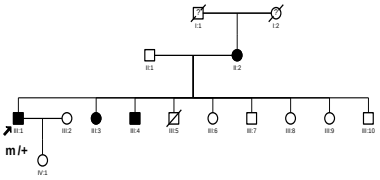

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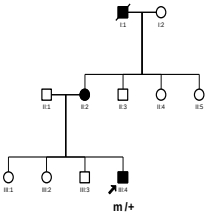

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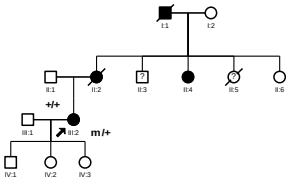

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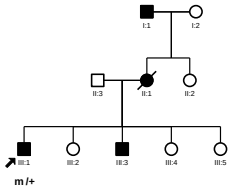

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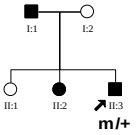

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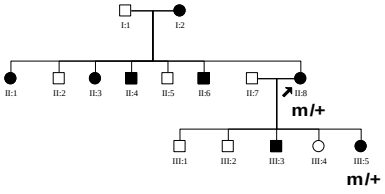
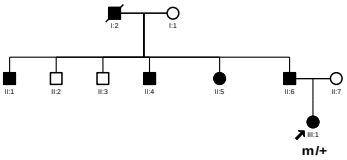
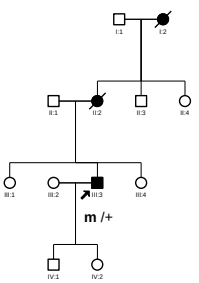


RP-2021
m: *RHO* p.Pro170Arg



RP-2179
m: *RHO* p.Gln28His



RP-2181
m: *RHO* p.Pro347Leu

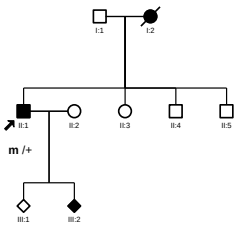


Figure S2. Two examples of electroretinogram (ERG) recordings. The probandus of RP-0640 and RP-2021.

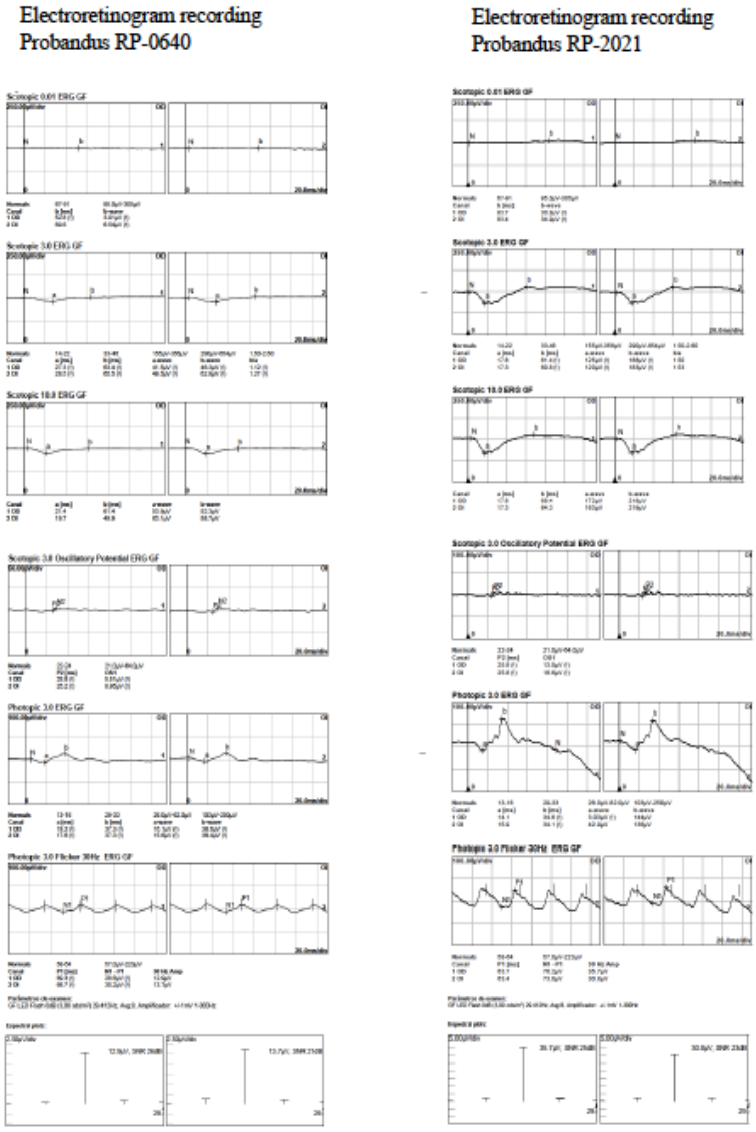


Table S1. (A) Mutation detection rates and screening methods. (B) Comparison of screening methods. The mutation detection rate increases, as expected, with the addition of other screening methods and when the results of the AD-RP microarray is considered for the 17 adRP genes, but not only for *RHO*. The number (or the fraction) of samples analysed by each method is given in parentheses.

Initial method	Increase of mutation detection rate with additional methods			Mutation detection rate for 17 adRP genes
SSCP (n)	**Added screening with SSCP and DGGE (n)**	**Added screening with SSCP, DGGE and AD-RP chip for *RHO* (n)**	**Added screening with SSCP, DGGE, AD-RP chip and full *RHO* sequencing (n)**	**Added screening with SSCP, DGGE and AD-RP chip for 17 adRP genes (n)**
5.9% (2/34)	13.0% (3/23)	0% (0/17)	8.3% (1/12)	11.8% (2/17)
DGGE (n)		**Added screening with DGGE and AD-RP chip for *RHO* (n)**	**Added screening with DGGE, AD-RP chip and full *RHO* sequencing (n)**	**Added screening with DGGE and AD-RP chip for 17 adRP genes (n)**
19.0% (16/84)		1.7% (1/60)	3.3% (1/30)	10.0% (6/60)
Chip AD-RP for *RHO* (n)			**Added screening with AD-RP chip and full *RHO* sequencing (n)**	**Added screening with AD-RP chip for 17 adRP genes (n)**
10.8% (18/167)			7.0% (6/85)	20.4% (34/167)
Full *RHO* sequencing (n)	!!			
7.0% (6/86)	!!			

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Table S2. Clinical data of patients presenting mutation in the Rhodopsin gene.

Mutation	Family	Pedigree	Night blindness Yes/No (years)	Visual field loss Yes/No (years)	Visual acuity loss Yes/No (years)	Age at diagnosis	Age(years) at time of testing	VA RE	LA RE	Visual field	Fundus	Electroretinogram (ERG)			
												Rods	Mixed	Cones	Flicker
p.Asn15Ser	RP-1927	III:2	Yes (18)	Yes(20)	No (45)	45	45	1	1	Tubular	Typical RP	NA	NA	NA	NA
		I:1	Yes (25)	Yes (25)	Yes (75)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		II:2	Yes (20)	Yes (20)	Yes (70)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Thr17Met	RP-0706	III:10	Yes (35)	Yes (40)	Yes (35)	22	NA	0,1	0,2	Tubular	NA	NA	NA	NA	NA
p.Glnr28His	RP-2179	III:3	Yes	Yes (14)	No (52)	35	52	1	1	Tubular	NA	NA	NA	NA	NA
p.Met44Thr	RP-0041	VI:13	Yes (15)	Yes (20)	Yes (26)	20	50	<0,1	<0,1	Absolute scotoma	Typical RP	NR	NR	NR	NR
p.Thr58Arg	RP-0820	III:1	Yes (10)	Yes (51)	Yes (40)	54	54	0,3	0,5	Tubular	Typical RP	NR	NR	NR	NR
		III:2	Yes (12)	Yes (50)	Yes (40)	50	NA	NA	NA	NA	NA	NA	NA	NA	NA
		II:1	Yes (25)	Yes	Yes (25)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Thr58Arg	RP-1908	III:4	Yes (10)	Yes (10)	Yes	31	31	0,6	0,5	Tubular	Typical RP	VD	VD	VD	VD
p.Leu79Pro	ONCE-0169	II:2	Yes (40)	Yes (50)	Yes (55)	57	NA	NA	NA	NA	NA	NA	NA	NA	NA
		I:2	Yes (50)	Yes (50)	Yes (50)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Thr97Ile	RP-0890	III:2	Yes (20)	Yes (40)	Yes	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Gly106Arg	RP-0242	III:14	Yes (9)	Yes (12)	Yes (32)	14	35	0,1	0,15	Tubular + islands	Typical RP	D	D	N	N
		II:5	Yes (45)	Yes (45)	Yes (45)	69	69	0,1	0,2	Tubular	NA	NA	D	D	D
		III:11	Yes (41)	Yes (42)	Yes (42)	41	42	1	0,2	NA	Typical RP	N	D	N	N
		III:1	Yes (34)	Yes (34)	Yes (34)	40	40	0,5	0,3	Superior scotoma	Typical RP	D	N	N	N
		III:3	Yes (32)	Yes (32)	Yes (32)	37	37	0,5	0,5	Superior scotoma	Typical RP	D	N	N	N
		IV:3	No (8)	No (8)	No (8)	8	8	1	1	NA	N	D	D	D	D
p.Gly106Arg	RP-0515	IV:1	Yes	Yes	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Trp126*	RP-1584	III:12	Yes (6)	Yes (6)	Yes (18)	25	26	0,5	0,5	Peripheral constriction :50º	NA	NR	NR	NR	NR
p.Arg135Trp	RP-0314	III:5	Yes	Yes	Yes	52	52	0,1	0,3	Tubular	Typical RP	VD	VD	VD	VD
		II:2	Yes	Yes	Yes	84	84	0,1	0,1	Absolute scotoma	Typical RP	NR	NR	NR	NR
		III:2	Yes	Yes	Yes	55	55	0,1	0,1	Tubular	Typical RP	VD	NA	NA	NA
		IV:1	Yes (17)	Yes	NA	17	17	NA	NA	Tubular	Typical RP	NR	NR	NR	NR
		IV:6	Yes	Yes	Yes	26	26	NA	NA	Peripheral constriction	Typical RP	VD	NA	NA	NA
		IV:7	Yes	Yes	No (22)	22	22	1	1	Peripheral constriction	Typical RP	VD	NA	NA	NA
p.Arg135Leu	RP-1188	III:2	Yes (3)	Yes (3)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Arg135Leu	RP-1876	III:1	Yes (1)	Yes (15)	Yes (1)	20	27	0,5	0,5	Tubular	Typical RP	NA	NA	NA	NA
p.Met163Thr	RP-0269	II:1	Yes (20)	Yes (22)	Yes (40)	38	54	0,17	0,25	Tubular	NA	NA	NA	NA	NA
p.Ala164Glu	RP-0727	III:3	Yes (37)	Yes (30)	Yes (37)	37	40	0,1	0,1	Tubular	Typical RP	NA	NA	NA	NA
p.Ala164Glu	RP-1930	III:1	Yes	Yes	NA	NA	48	0,2	0,3	Peripheral constriction: 30º	Typical RP	NA	VD	D	NA
p.Cys167Tyr	RP-1458	III:1	Yes (8)	Yes (25)	Yes (30)	35	58	0,2	0,2	Tubular	Typical RP	NR	NR	NR	NR
p.Cys167Tyr	RP-1941	II:3	Yes (7)	Yes (35)	Yes (40)	40	40	0,6	0,4	Tubular	NA	NA	NA	NA	NA
p.Pro170Arg	RP-2021	III:1	Yes (15)	Yes (15)	No (17)	17	17	1	0,9	NA	N	VD	D	N	N
		II:6	Yes (15)	Yes (15)	Yes (44)	16	NA	NA	NA	NA	NA	NA	NA	NA	NA
		II:1	Yes (15)	Yes	Yes	15	NA	NA	NA	NA	NA	NA	NA	NA	NA
		II:4	Yes (15)	Yes	Yes	17	NA	NA	NA	NA	NA	NA	NA	NA	NA
		II:5	Yes (15)	Yes	Yes	26	NA	NA	NA	NA	NA	NA	NA	NA	NA
		I:2	Yes (19)	Yes (19)	Yes	19	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Pro171Leu	RP-1125	III:1	Yes (10)	Yes (10)	No (38)	15	38	1	1	Tubular	Typical RP	NA	NA	NA	NA
		II:2	Yes (10)	Yes (10)	Yes	60	60	0,5	0,7	Tubular	NA	NA	NA	NA	NA
		III:3	Yes (10)	Yes (3)	No (23)	3	23	1	1	Tubular	Typical RP	NA	NA	NA	NA
		III:2	Yes (10)	Yes (10)	Yes	13	34	0,8	0,7	Tubular	NA	NA	NA	NA	NA
IVS2 as A-G -2	RP-0568	V:4	Yes (17)	Yes (7)	Yes (30)	17	38	0,4	0,6	Tubular + temporal island	Typical RP	NA	NA	NA	NA
		IV:4	Yes	Yes	Yes (64)	73	74	0,5	<0.1	Peripheral constriction	Typical RP	NA	NA	NA	NA
		V:1	Yes (15)	Yes (40)	Yes (45)	40	43	0,1	0,1	RE: absolute scotoma LE: tubular	Typical RP	NA	NA	NA	NA
p.Tyr178Cys	RP-0913	III:1	Yes (6)	Yes (12)	Yes	10	36	0,6	LP	Tubular	Typical RP	NR	NR	NR	NR
		III:3	Yes (15)	Yes	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		II:7	Yes (10)	Yes	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Glu181Lys	RP-1672	III:2	Yes (5)	Yes (6)	Yes (16)	10	57	0,2	0,2	Tubular	Typical RP	NA	NA	NA	NA
p.Gly182Ser	RP-0640	III:9	Yes (8)	Yes (45)	Yes (50)	45	52	0,5	0,5	Absolute scotoma superior hemirretina	Typical RP	NR	VD	D	NA
		IV:3	Yes	Yes	No (26)	26	26	1	1	pheripheral scotoma	NA	D	D	N	N

		III:3	Yes (8)	Yes (18)	No (18)	18	18	1	1	pheripheral scotoma	NA	NA	NA	NA	NA
p.Gly182Ser	RP-1375	III:1	Yes (13)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		III:2	Yes (10)	Yes (10)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Gly182Val	RP-1952	II:8	Yes (6)	Yes (45)	Yes (45)	45	45	0,02	0,02	Tubular	NA	NA	NA	NA	NA
p.Ser186Pro	RP-0115	III:3	Yes (15)	Yes (15)	Yes (23)	20	23	0,5	0,4	Peripheral constriction: 20º-30º	Typical RP	NR	D	D	NR
		II:4	Yes (3)	Yes (3)	Yes (25)	6	43	0,4	0,4	Tubular + temporal island	Typical RP	NR	NR	NR	NR
		IV:2	Yes (27)	Yes (32)	Yes (33)	40	60	LP	LP	Absolute scotoma	NA	NR	NR	NR	NR
		V:1	Yes (5)	Yes (32)	Yes (36)	30	36	1	0,2	Tubular + inferior island	Typical RP	NR	VD	VD	N
		V:3	Yes (10)	Yes (15)	Yes	15	34	0,7	0,3	Peripheral constriction: 30º	Typical RP	NR	VD	VD	NA
p.Gly188Arg	RP-0352	V:4	Yes (5)	Yes (25)	Yes (29)	25	29	0,3	0,8	Tubular + relatives diffuses scotomas	Typical RP	NR	D	N	NA
		V:5	Yes (7)	Yes (23)	Yes (23)	23	27	0,15	0,7	Tubular	Typical RP	NR	D	D	NA
		VI:1	Yes (3)	No (4)	Yes	4	4	0,2	0,2	NA	NA	NA	NA	NA	NA
		VI:2	No (3)	No (3)	No (3)	3	3	NA	NA	NA	NA	NA	NA	NA	NA
		IV:6	Yes (8)	Yes (18)	Yes	14	51	0,7	0,7	Tubular + island	Typical RP	NR	NR	NR	NR
		IV:14	Yes (10)	Yes (14)	Yes (41)	44	44	1	0,2	Tubular + island	Typical RP	NR	NR	NR	NR
		V:14	Yes (4)	Yes (10)	Yes (14)	4	17	0,5	0,4	Temporal scotoma	Typical RP	NR	NR	NR	NR
		V:15	No (4)	No (4)	Yes	4	4	0,2	0,2	NA	Typical RP	NA	NA	NA	NA
p.Asp190Tyr	RP-0188	IV:9	Yes (18)	Yes (18)	Yes (42)	NA	47	<0,1	0,5	Temporal scotoma	Typical RP	NR	NR	NR	NR
		V:8	Yes (15)	Yes	Yes	15	16	0,5	0,6	Superior scotoma	Typical RP	NA	NA	NA	NA
		III:2	Yes (20)	Yes (18)	Yes (45)	20	80	<0,1	<0,1	NA	Typical RP	NR	NR	NR	NR
		IV:11	Yes (7)	Yes (14)	Yes (30)	14	43	0,8	0,6	Peripheral constriction + scotoma	Typical RP	NR	NR	NR	NR
		III:2	Yes (18)	Yes (30)	Yes (40)	20	55	<0,1	<0,1	Tubular	Typical RP	NR	NR	NR	NR
		IV:7	Yes (10)	Yes (10)	NA	37	37	LP	0,5	Peripheral constriction	Typical RP	NR	NR	NR	NR
		V:4	Yes (6)	Yes (12)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Asp190Tyr	RP-0328	IV:1	Yes (19)	Yes (25)	Yes (30)	9	NA	NA	NA	NA	NA	NA	NA	NA	NA
		IV:5	Yes (15)	Yes	Yes	17	20	0,3	0,3	Tubular	Typical RP	NR	NR	NR	NR
		IV:3	Yes (20)	Yes	NA	26	26	0,8	1	Peripheral superior + temporal scotoma	NA	NR	NR	D	D
p.Pro215Leu	RP-0566	II:3	Yes (10)	Yes (10)	Yes (15)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Thr289Pro	RP-0206	II:1	Yes (25)	Yes (45)	Yes (43)	54	71	0,2	0,1	Tubular + islands	NA	NA	NA	NA	NA
		IV:2	Yes (10)	Yes	Yes	8	38	0,4	0,4	Tubular	Typical RP	NR	NR	NR	NR
		III:2	Yes (7)	Yes(7)	Yes (24)	7	55	<0,1	<0,1	Absolute scotoma	Typical RP	NR	NR	NR	NR
		III:3	Yes (10)	Yes (10)	Yes (20)	10	51	<0,1	<0,1	Tubular	Typical RP	NR	NR	NR	NR
		IV:7	Yes (10)	Yes (10)	Yes	10	19	0,7	0,7	Tubular	Typical RP	NR	NR	NR	NR
p.Pro347Leu	RP-0220	IV:8	Yes (7)	Yes (7)	Yes	7	16	0,3	0,3	Tubular	Typical RP	NA	NA	NA	NA
		IV:9	Yes (8)	Yes (15)	Yes(24)	8	27	0,1	0,1	Tubular	Typical RP	NR	NR	NR	NR
		IV:11	Yes (7)	Yes	Yes	7	21	0,3	0,3	Tubular	Typical RP	NR	NR	NR	NR
		V:2	Yes (10)	Yes	Yes	10	11	0,7	0,7	Tubular	Typical RP	NR	VD	D	NA
p.Pro347Leu	RP-0542	III:13	Yes (12)	Yes (17)	Yes (17)	17	35	0,5	0,5	Tubular	Typical RP	NR	NR	NR	NR
p.Pro347Leu	RP-0685	III:2	Yes (14)	Yes (14)	Yes	14	37	0,4	0,7	Tubular	Typical RP	NA	NA	NA	NA
p.Pro347Leu	RP-0900	III:8	Yes (13)	Yes (15)	Yes (25)	NA	41	0,3	0,3	NA	Typical RP	NA	NA	NA	NA
		III:6	Yes (15)	Yes (15)	Yes (25)	16	NA	NA	NA	NA	NA	NA	NA	NA	NA
		IV:1	Yes (10)	Yes (10)	Yes (10)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Pro347Leu	RP-0975	III:3	Yes (10)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		II:1	Yes (10)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		II:3	Yes (20)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
		IV:1	Yes (2)	Yes (2)	Yes	8	10	0,5	0,5	Tubular	Typical RP	NR	NA	D	NA
p.Pro347Leu	RP-1453	III:7	Yes (6)	No (40)	Yes	7	40	0,2	0,2	NA	NA	NA	NA	NA	NA
		IV:2	Yes (7)	Yes (7)	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
p.Pro347Leu	RP-1472	IV:2	Yes (4)	Yes (4)	Yes (4)	NA	47	0,2	0,2	Tubular	Typical RP	NA	NA	NA	NA
		V:1	Yes (10)	Yes (10)	Yes (10)	10	23	0,8	0,8	Tubular	Typical RP	NR	NR	NR	NR
		III:2	Yes (1)	Yes (4)	Yes (35)	4	52	NA	NA	Tubular + paranasal island	Typical RP	NR	NR	NR	NR
p.Pro347Leu	RP-1697	IV:2	Yes (2)	Yes (16)	Yes (30)	4	40	0,3	0,3	Peripheral constriction :15º	Typical RP	NR	NR	NR	NR
		IV:3	Yes	Yes	Yes	34	36	0,6	0,5	Tubular	Typical RP	NR	NR	NR	NR
p.Pro347Leu	RP-2181	II:1	Yes (3)	Yes	Yes (40)	12	NA	NA	NA	NA	NA	NA	NA	NA	NA

Legends: RP: Retinitis Pigmentosa; NA: Not Available; NR: Non Recordable; VD: Very Diminished; D: Diminished; N: Normal; VA: Visual Acuity; RE: Right Eye; LE: Left Eye

Table S3. Age at onset of symptoms and diagnosis of all mutated patients, patients with p.Pro347Leu mutation and patients with other *RHO* mutations. *Number of patients with statistical calculations. **Comparisons were performed using the t- Student test.

	NIGHT BLIDNESS				VISUAL FIELD LOSS				VISUAL ACUITY LOSS				DIAGNOSIS		
	Present	N*	Average Age of onset ± SD	*p-value***	Present	N*	Average Age of onset ± SD	*p-value***	Present	N*	Average Age of onset ± SD	*p-value***	N*	Average Age ± SD	*p-value***
Patients with p.Pro347Leu mutation	25/25 100.0%	24	9 ± 4.5 1 to 20 years	0.0001	21/22 95.4%	16	10 ± 4.7 2 to 16 years	0.0004	21/21 100.0%	12	22 ± 10.5 4 to 40 years	0.0008	18	11 ± 6.8 1 to 34 years	< 0.0001
Patients with other *RHO* mutations	79/82 96.3%	69	15 ± 10.6 1 to 45 years		78/82 95.1%	58	23 ± 13.9 2 to 51 years		61/71 86.0%	42	36 ± 14.5 1 to 75 years		67	28 ± 18.2 1 to 45 years	
All mutated patients	104/107 97.2%	93	14 ± 9.9 1 to 45 years	-	99/104 95.2%	74	20 ± 13.6 2 to 51 years	-	82/92 89.1%	54	33± 14.9 1 to 75 years	-	85	24 ± 18.0 1 to 54 years	-

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CAPÍTULO IV

Caracterización genética de pacientes con adRP utilizando secuenciación masiva mediante paneles de genes

Artículo*: Targeted next generation sequencing improves the diagnosis of autosomal dominant Retinitis Pigmentosa in Spanish patients*

En los últimos años, el desarrollo de métodos de secuenciación masiva ha transformado nuestra capacidad para diagnosticar enfermedades hereditarias. Los estudios demuestran que son herramientas rentables para el diagnóstico molecular de enfermedades con elevada heterogeneidad clínica y genética, como es el caso de las distrofias de retina.

Con el fin de implementar y aplicar una nueva herramienta de *NGS* centrada en el diagnóstico de pacientes con distrofia de retina, en nuestro laboratorio se desarrolló una estrategia basada enla secuenciación dirigida mediante un panel de 73 genes asociados a formas de RP y LCA. En este trabajo se presenta su evaluación en una cohorte de 59 pacientes diagnosticados clínicamente de adRP, que previamente habían sido estudiados, conreultado negativo, mediante otras herramientas genéticas clásicas siguiendo el algoritmo descrito en el anterior capítulo. En el momento de su publicación, este trabajo recogía la mayor cohorte de pacientes con adRP estudiada mediante estrategias de *NGS* en población occidental y la segunda mayor a nivel mundial.

Se siguió un protocolo de priorización de variantes específico para este grupo de pacientes seleccionando variantes en los exones y regiones flanqueantes descritas como causantes de enfermedad en bases de datos y variantes nuevas que fueron filtradas por frecuencia del alelo minoritario (*Minor Allele Frequency, MAF*) ≤ 0,001 en diferentes bases de datos poblacionales. Se consideraron patogénicas los siguientes tipos de variantes detectadas: i) variantes nuevas y poco frecuentes que causan el truncamiento de proteínas (*loss-of function, LOF; nonsense y frameshift*); ii) las variantes cuyo análisis *insilico* predecía un efecto perjudicial sobre el *splicing* del ARNm; iii) *missense* predichas como patogénicas en al menos dos de las tres herramientas *in silico* utilizadas. Se priorizaron variantes en heterocigosis en genes previamente asociados con adRP y en última instancia, cuando no se encontraron variantes candidatas en los pasos anteriores, también se consideraron variantes asociadas con otros patrones de herencia (AR o XL) o presentaciones clínicas.

De este modo se identificaron un total de 31 variantes candidatas en 28 familias que fueron clasificadas, según su nivel demostrado de patogenicidad, en tres categorías. Primero, se consideraron 17 Variantes Causales, incluyendo seis mutaciones conocidas asociadas a DR, seis variantes *LOF* nuevas y cinco *missensse* nuevas. Otros siete cambios *missense* e *in-frame* fueron clasificados como Variantes de Significado Incierto (*Variant of Uncertain Significance,*

VUS), las cuales, se predijeron como patogénicas con herramientas *in silico* y estaban ausentes en 300 alelos de población control españolaen la que no se pudo realizar una cosegregación en las familias o ésta no fue concluyente por falta de informatividad del árbol familiar. Por último, siete variantes fueron clasificadas como probablemente no causantes debido a que no cosegregaron con la patología en sus respectivas familias y / o su presencia en al menos uno de los 300 alelos de la población de control (MAF: 0,003).

Finalmente con esta aproximación de secuenciación dirigida se caracterizaron 16 de 59 familias, permitiendo una tasa diagnóstica del 27%. Dos tercios de las familias caracterizadas presentaron mutaciones nuevas, y el tercio restante presentó mutaciones conocidas. Las mutaciones encontradas se localizaron preferentemente en los genes asociados a patología dominante *RP1*, *PRPF31*, *PRPH2*, *SNRNP200*, *CRX*, *GUCA1B*, *IMPDH1* y *RHO*. Además, el 5% de las familias caracterizadas (3 de 59) fueron reclasificadas genéticamente al identificarse variantes patogénicas en *ABCA4,* gen asociado a formas recesivas de DCB y en los genes *RPGR* y *RP2* asociados a formas ligadas al cromosoma X.

Esta estrategia de secuenciación masiva mediante paneles de genes ha demostrado ser efectiva para el diagnóstico de pacientes con adRP, aportando información relevante sobre las mutaciones encontradas, permitiendo proporcionar un diagnóstico preciso y un adecuado asesoramiento genético a los pacientes y sus familiares, además de aumentar el conocimiento de la patogénesis de estas formas de RP e incrementar la tasa diagnóstica respecto a las técnicas convencionales.

Aportación de la autora

En este trabajo la autora estuvo implicada en el diseño y la recopilación retrospectiva de los datos genéticos de las 59 familias analizadas. Además, generó las librerías mediante hibridación y captura con el sistema de enriquecimiento *Haloplex* y llevó a cabo el análisis bioinformático, filtrando y priorizando las variantes e interpretando los resultados obtenidos. Posteriormente, se encargó de validar y segregar mediante secuenciación Sanger en un total de 97 individuos las 31 variantes candidatas detectadas en este estudio. También realizó el estudio de 15 variantes en 150 controles mediante la técnica de *HRM* (*High ResolutionMelting*) y/o secuenciación Sanger. Por otro lado, colaboró en la revisión de la información clínica para realizar correlaciones fenotipo-genotipo y, finalmente, participó en la escritura de un primer borrador para la publicación de este trabajo.

Genetics

Targeted Next-Generation Sequencing Improves the Diagnosis of Autosomal Dominant Retinitis Pigmentosa in Spanish Patients

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Purpose. Next-generation sequencing (NGS) has been demonstrated to be an effective strategy for the detection of mutations in retinal dystrophies, a group of inherited diseases that are highly heterogeneous. Therefore, the aim of this study is the application of an NGS-based approach in a Spanish cohort of autosomal dominant retinitis pigmentosa (RP) patients to find out causative mutations.

Methods. Index cases of 59 Spanish families with initial diagnosis of autosomal dominant RP and unsuccessfully studied for mutations in the most common RP causal genes, were selected for application of a NGS-based approach with a custom panel for 73 genes related to retinal dystrophies. Candidate variants were select based on frequency, pathogenicity, inherited model, and phenotype. Subsequently, confirmation by Sanger sequencing, cosegregation analysis, and population studies, was applied for determining the implication of those variants in the pathology.

Results. Overall 31 candidate variants were selected. From them, 17 variants were considered as mutations causative of the disease, 64% (11/17) of them were novel and 36% (6/17) were known RP-related mutations. Therefore, applying this technology16 families were characterized rendering a mutation detection rate of 27% (16/59). Of them, 5% (3/59) of cases displayed mutations in recessive or X-linked genes (*ABCA4*, *RPGR*, and *RP2*) allowing a genetic and clinical reclassification of those families. Furthermore, seven novel variants with uncertain significance and seven novel variants probably not causative of disease were also found.

Conclusions. This NGS strategy is a fast, effective, and reliable tool to detect known and novel mutations in autosomal dominant RP patients allowing genetic reclassification in some cases and increasing the knowledge of pathogenesis in retinal dystrophies.

Keywords: target NGS, retinitis pigmentosa, autosomal dominant, Spanish families

Retinitis pigmentosa (RP), with a prevalence of 1:4000,[1] is the most common form of inherited retinal dystrophy (RD) affecting mainly rod photoreceptors and inducing a progressive vision loss.[2] The disease is characterized by an extraordinary genetic and clinical heterogeneity. Clinical symptoms include night blindness, progressive loss of peripheral vision, decreased visual acuity in late stages, abnormal retinal electrophysiology, and ocular fundus alterations.

Genetic heterogeneity of this disorder includes classical monogenic mode of inheritance (autosomal dominant, autosomal recessive, and X-linked transmission) as well as digenic and mitochondrial inherited patterns. Autosomal dominant retinitis pigmentosa (adRP) accounts for approximately 15% of Spanish RP families.[3] To date, more than 70 genes have been associated with nonsyndromic RP and 25 of them have been identified related to adRP (Retnet, updated November 2014; available in the public domain, https://sph.uth.edu/retnet/).

Genetic analysis of these patients is complicated because of the large number of genes involved in adRP disease, the presence of incomplete penetrance in some families and a high inter- and intrafamilial phenotypic heterogeneity exhibited by some of them.[2,4]

Recently, targeted next-generation sequencing (NGS) has become an effective tool for determining the causal mutation in adRP as demonstrated by different reports.[5–11] Therefore, we have developed a specific strategy based on DNA capture of coding and noncoding regions of 73 genes associated with RP

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and Leber congenital amaurosis (LCA) that we are applying to the diagnosis of uncharacterized RD patients.

Because it has been estimated that approximately 80% of adRP families have mutations in known genes,[12] the current study proposes the use of a customized NGS panel of RD genes in a large cohort of adRP Spanish families unsuccessfully studied with classical tools.

Methods

Subjects and Samples

Among 116 uncharacterized index patients out of 195 families with initial diagnosis of RP and likely autosomal dominant inheritance, 59 were selected to be included in the proposed NGS-based approach. Diagnosis of adRP was determined according to a dominant mode of inheritance in patients with night blindness, peripheral vision loss, and ocular fundus alteration and reduced scotopic response.[3] Clinical evaluation was assessed as previously described.[13]

Additionally, 150 healthy unrelated Spanish individuals without familial history of retinal dystrophy were screened as controls to evaluate the frequency of the novel missense and in-frame variants.

All families were recruited at the Fundacion Jimenez Diaz University Hospital (Madrid, Spain). This study was reviewed and approved by the ethics committee of the Hospital following the tenets of the Declaration of Helsinki and reviews. The participating subjects, or their legal guardians, signed a written informed consent form after the nature of procedures had been explained.

Genomic DNA was extracted from peripheral blood leukocytes using automated DNA extractors: BioRobot EZ1 (Qiagen, Hilden, Germany) and MagNA Pure Compact system (Roche Applied Science, Penzberg, Germany).

Molecular Methods

All 59 index patients had been previously screened using a combined strategy of several molecular tools: single-strand conformation polymorphism (SSCP), CG-clamped denaturing gradient gel electrophoresis (DGGE), genotyping ADRP Chip (AsperBiotech, Tartu, Estonia), long-range PCR followed by NGS, and Sanger sequencing of the most prevalent adRP genes[13–16] (Supplementary Table S1).

Design of RD Panel

A customized RD_NGS_Panel of 73 previously known RD genes reported in Retnet (available in the public domain at https://sph.uth.edu/retnet/) and literature was developed using the Haloplex capture technology (Agilent Technologies, Inc., Santa Clara, CA, USA; Supplementary Table S2).

First, genomic coordinates of coding and noncoding exons for all RefSeq transcripts (hg19) were obtained from the University of California Santa Cruz (UCSC; Santa Cruz, CA, USA) Genome Browser using the Galaxy software.[17]

Amplicons for all coding and noncoding exons, including 20 bp of flanking 5′ and 3′ intronic sequence were designed using the SureDesign tool (available in the public domain at https://earray.chem.agilent.com/suredesign/; Agilent Technologies, Inc.). Additionally four intronic regions for previously known deep intronic mutations were also included in the final design (Supplementary Table S3).

In total, 1127 regions were targeted comprising 352 kb of target sequence. The final design covered 99.1% of the requested target regions, as showed in Supplementary Table S2.

Sequence Capture and Next-Generation Sequencing

Target enrichment was performed according to HaloPlex Enrichment System for Illumina Sequencing protocol (version D3, December 2012; Agilent Technologies, Inc.) with some modifications: (1) genomic DNA starting material (450 ng) was digested in eight restriction reactions, (2) digested fragments were hybridized to the complementary HaloPlex probe capture library. In this step, index sequences for multiplexing were incorporated into the targeted fragments, (3) DNA-HaloPlex probe hybrids containing biotin were captured on streptavidin beads and ligation of fragments was performed, (4) captured target libraries were amplified by PCR, and (5) amplified products were purified twice with AMPure XP beads (Beckman Coulter, Brea, CA, USA) and additionally, the number of washes with EtOH was doubled. Its concentrations were determined using a 2100 Bioanalyzer (Agilent Technologies, Inc.). Captured target libraries from 59 probands were pooled and finally sequenced using MiSeq v2 reagents kit (Illumina, San Diego, California, USA) on six runs in an Illumina MiSeq system to obtain 150 bp paired-end reads. The base calling and quality control were performed using Illumina RTA sequence analysis pipeline.

Bioinformatic Analysis

A specific custom pipeline for HaloPlex kits on Illumina implemented into the commercial DNAnexus platform (available in the public domain at https://www.dnanexus.com/) was used for the bioinformatic analysis (Fig. 1). After trimming the adapter sequence, reads from the Illumina MiSeq instrument were mapped against the hg19 human reference genome using Burrows-Wheeler Aligner (BWA)-MEM version 0.7.5a with default parameters to generate the mappings.[18] Genome Analysis Toolkit(GATK)-lite version 2.3-9 was applied for local realignment, recalibration of base quality scores and variant calling.[19,20] Reads and coverage statistics were produced using PRINSEQ-lite version 0.20, Picard CalculateHsMetrics version 1.97 (available in the public domain at www.picard.sourceforge.net), and BedTools Coverage version 2.17.0 tools.[21,22] Finally, GATK Variant Annotator and ENSEMBL Variant Effect Predictor v72 tools were used for variant annotation and creation of vCard files[23] (Fig. 1).

Appraisal of Sensitivity

Twenty-nine known variants previously detected by Sanger sequencing in 16 of 59 cases were used as control samples to evaluate the technology applied (Supplementary Table S4).

Variant Priorization and Pathogenicity Assessment

The following criteria were considered for variant filtering (Fig. 1): (1) variants were selected focusing on known, novel, and rare variants in exome target excluding intergenic or intronic sequence. The former were considered directly as pathogenic using the Human Gene Mutation Database (HGMD Professional; available in the public domain at www.biobase-international.com/product/hgmd) and rare variants were filtered by a minor allele frequency (MAF) less than or equal to 0.001 in the 1000 Genomes (available in the public domain at www.1000genomes.org/data), dbSNP132 (in the public domain, http:// www.ncbi.nlm.nih.gov/projects/SNP), and Exome Variant Server (EVS; available in the public domain at http://evs.gs.washington.edu/EVS/) databases, (2) novel and rare variants causing protein truncation such as nonsense and frameshift were considered as pathogenic, (3) then, variants in silico predicted as having

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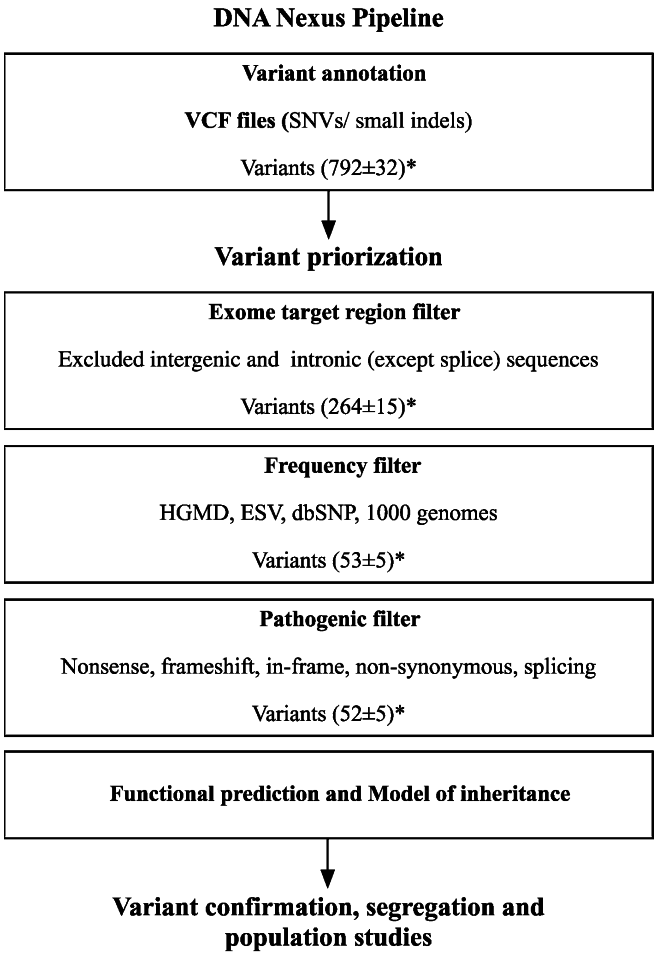

FIGURE 1. Pipeline used to identify and validate candidate variants. *Average and SD per case.

deleterious effect on mRNA splicing as analyzed by the Human Splicing Finder version 2.4 (available in the public domain at www.umd.be/HSF) software tool were also incorporated, (4) nonsynonymous single nucleotide variants (SNVs) predicted as damaging in at least two of three in silico tools (SIFT,[24] Mutation Taster,[25] and PolyPhen-2[26]) used for pathogenicity prediction were also included; and (5) heterozygous variants in genes previously associated with adRP were considered but when no candidate variants were found in the above steps, variants associated with different RD patterns and/or clinical presentations were also included.

All candidate causal variants identified by NGS were further verified by Sanger sequencing on an ABI 3130 Genetic Analyzer sequencer (Applied Biosystems, Foster City, CA, USA). Cosegregation analysis of each variant that was suspected as causal RP phenotype was performed using available DNA from affected and unaffected family members assuming in most cases an autosomal dominant trait model with no phenocopies and complete penetrance. All candidate variants were screened in 150 alleles from 75 in-house whole exomes Spanish control individuals (available in the public domain at http://bioinfo.cipf.es/apps-beta/exome-server/beta/). Additionally, novel, nonsynonymous, and in-frame variants were screened in another 150 Spanish control chromosomes by Sanger sequencing and high resolution melting (HRM) as previously reported.[27]

Finally, candidate causal variants were classified into three categories according to their demonstrated level of pathogenicity, as shown in Figure 2: (1) Variants causative of the disease, including known RD mutations, novel loss of function

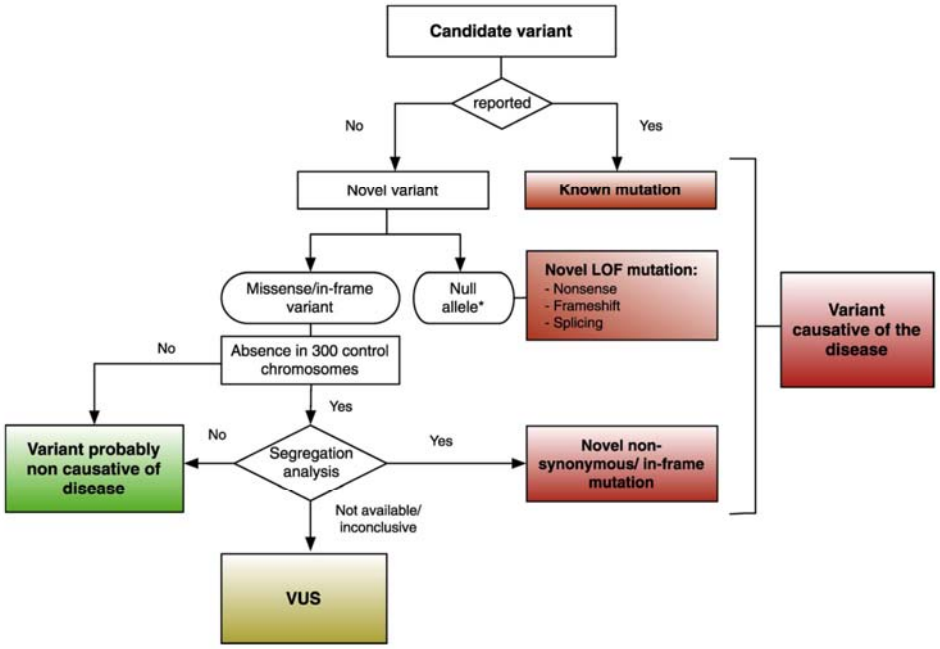

FIGURE 2. Algorithm applied for classification of candidate variants. *Cosegregation analysis was performed, when possible.

(LOF), nonsynonymous and in-frame mutations, (2) variants with uncertain significance (VUS): nonsynonymous and in-frame rare variants predicted as pathogenic with in silico tools, absent in 300 Spanish control population alleles and with unavailable or inconclusive cosegregation testing, and (3) variants probably not causative of disease (rare variants excluded as responsible of the disease through cosegregation studies and/or presence in at least one of 300 control population alleles (MAF: 0.003).

RESULTS

Analysis of Target NGS Approach

A custom RD_NGS_Panel for 73 genes was applied in 59 adRP index patients unsuccessfully studied by classical genetic tools. A total of 1236 regions were covered.

A mean of 2,915,152 reads in binary alignment/map (BAM) files was generated per sample with an average read length of 130 bp. From them, 2,217,542 reads (76.07%) were in analyzable target regions with an average and median read depth of 606 and 512, respectively. The 0.5% of target regions were covered less than 20 reads, and the 98.1% and 84.7% were covered by 100 and 500 reads, respectively.

Additionally, in order to check the reliability of our approach, 29 known variants in six different genes, previously detected by Sanger sequencing in 16 patients, were used as control samples and all of them were redetected by this technology (Supplementary Table S4).

A mean of 792 (±32) SNVs and indels were identified per sample. Removing off-target variants as intergenic or intronic resulted in a mean of 264 (±15) variants. Filtering by frequency data, a mean of 53 (±5) rare variants were selected. Removing synonymous and noncoding variants, a mean of 52 (±5) variants remained per sample (Fig. 1). Finally, considering an autosomal dominant model of inheritance and in silico prediction analysis, we identified a total of 31 candidate variants in 28 families. Of them, 68% (21/31) were missense variants, 10% (3/31) were frameshift changes, 10% (3/31) were stop codon mutations, 6% (2/31) were SNVs located in splice sites, and 6% (2/31) were in-frame deletions. All these variants were confirmed by Sanger sequencing. Additionally cosegregation analysis and population studies were performed in order to identify potential pathogenic mutations (Supplementary Figs. S1, S2).

Identification of Mutations and Spectrum of Variants

Known mutations previously reported related to adRP were identified in four families: *CRX* (p.Ala198Glyfs*38), *PRPF31* (c.1146+2T>C), *RHO* (p.Asn15Ser), and *SNRNP200* (p.Ser1087Leu), as shown in Table 1. Additionally, five families displayed novel putative LOF protein mutations: two nonsense mutations in *RP1* (p.Glu661* and p.Tyr915*); two frameshift variants: in *PRPF31* (p.Gly314Argfs*10) and *RP1* (p.Asn763Ilefs*12), and last, one splicing mutation in *PRPH2* (c.582-1G>A).

Moreover, another four families carried novel nonsynonymous mutations in *GUCA1B* (p.Arg44His), *IMPDH1* (p.Ala321V-

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Table 1. Spectrum of Variants Causative of the Disease Identified With the RD_NGS_Panel in Autosomal Dominant Retinitis Pigmentosa Families

Family	Gene	NM	HGVS-cdna	HGVS-prot	Effect	Zyg	dbSNP (MAF)	SIFT	Polyphen-2	Mutation Taster	Human Splicing Finder	Cosegregation (Affected/ Unaffected/ Asymptomatic)	Frequency in Spanish Control Alleles	Reference
Known mutations														
RP-1875	*ABCA4**	NM_000350.2	c.3386G>T	p.Arg1129Leu	Missense	Het	rs1801269 (<0.01)	D (0)	Pr-D (0.992)	DC (1.000)	-	Yes (2/11)	0/150	Allikmets et al.,[28] 1997
			c.6148G>C	p.Val2050Leu	Missense	Het	rs41292677 (0.04)	D (0.01)	Pr-D (0.950)	DC (0.999)	-		0/150	Allikmets et al.,[29] 1997
RP-1688	*CRX*	NM_000554.4	c.586_587insC	p.Ala198Glyfs*38	Frameshift	Het	-	-	-	DC (1.000)	-	NA	0/150	Sohocki et al.,[30] 1998
RP-1970	*PRPF31*	NM_015629.3	c.1146+2T>C	-	Splicing	Het	-	-	-	DC (1.000)	Decrease 5′donor site of exon 11 (90.88–>72.59)	Yes (2)	0/150	Waseem et al.,[31] 2007
RP-0642	*RHO*	NM_000539.3	c.44A>G	p.Asn15Ser	Missense	Het	rs104893786 (nfd)	D (0)	Pr-D (0.998)	DC (1.000)	-	Yes (1/2)	0/150	Kranich et al.,[32] 1993
RP-1480	*SNRNP200*	NM_014014.4	c.3260C>T	p.Ser1087Leu	Missense	Het	-	D (0)	Pr-D (0.995)	DC (1.000)	-	Yes (4/4/4)	0/150	Zhao et al.,[33] 2009
Novel LOF protein mutations														
RP-1541	*PRPF31*	NM_015629.3	c.937_938insA	p.Gly314Argfs*10	Frameshift	Het	-	-	-	DC (1.000)	-	Yes (2/2)	0/150	This study
RP-1176	*PRPH2*	NM_000322.4	c.582-1G>A	-	Splicing	Het	-	-	-	-	Decrease 3′acceptor site of exon 2 (82.97–>54.03)	Yes (2)	0/150	This study
RP-2072	*RP1*	NM_006269.1	c.1981G>T	p.Glu661*	Nonsense	Het	-	-	-	DC (1.000)	-	NA	0/150	This study
RP-1890	*RP1*	NM_006269.1	c.2286delA	p.Asn763Ilefs*12	Frameshift	Het	-	-	-	DC (1.000)	-	Yes (2)	0/150	This study
RP-1387	*RP1*	NM_006269.1	c.2745_2749del	p.Tyr915*	Nonsense	Het	-	-	-	DC (1.000)	-	Yes (4/2)	0/150	This study
RP-0631	*RPGR**	NM_000328.2	c.1234C>T	p.Arg412*	Nonsense	Het	-	-	-	DC (1.000)	-	Yes (2)	0/150	This study
Novel nonsynonymous/in-frame mutations														
RP-1728	*GUCA1B*	NM_002098.5	c.131G>A	p.Arg44His	Missense	Het	-	D (0.004)	B (0.065)	DC (0.997)	-	Yes (2/1)	0/300	This study
RP-0422	*IMPDH1*	NM_000883.3	c.962C>T	p.Ala321Val	Missense	Het	-	D (0)	Pr-D (0.926)	DC (1.000)	-	Yes (3/11)	0/300	This study
RP-0652	*PRPH2*	NM_000322.4	c.536G>T	p.Trp179Leu	Missense	Het	-	D (0.010)	Pr-D (1.000)	DC (1.000)	-	Yes (2)	0/300	This study
RP-0948	*RP1*	NM_006269.1	c.4328G>A	p.Arg1443Gln	Missense	Het	-	D (0)	Pr-D (0.974)	P (0.993)	-	Yes (2/1)	0/300	This study
RP-1682	*RP2**	NM_006915.2	c.9_11del	p.Phe4del	In-frame	Het	-	-	-	DC (0.986)	-	Yes (1/3)	0/300	This study

LOF, loss of function; Zyg, zygosity; Het, heterozygosis; nfd, no frequency data; SIFT, deleterious (D); Polyphen2: probably damaging (Pr-D) and benign (B); Mutation Taster: disease causing (DC) and polymorphism (P); NA, not available.

* Gene associated with other inherited model different to dominant pattern.

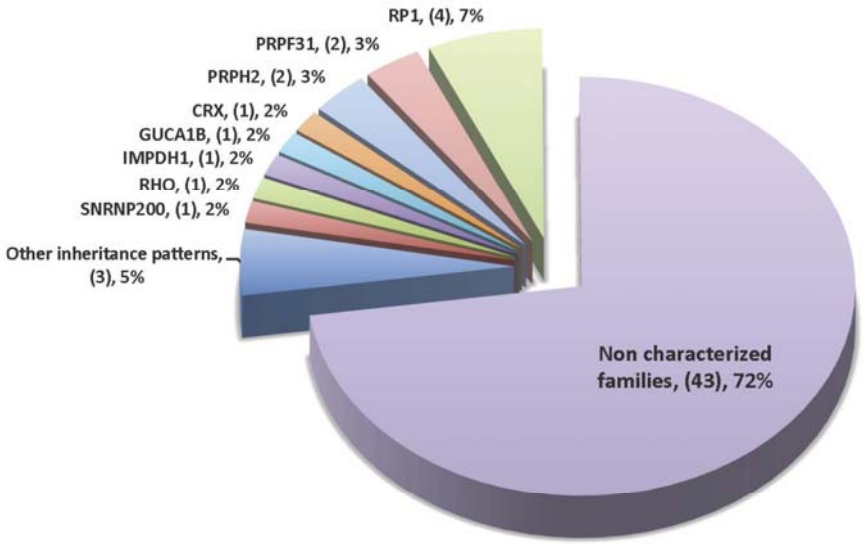

FIGURE 3. Percentage of families characterized with RD_NGS_Panel and spectrum of genes implicated in a cohort of 59 adRP families.

al), *PRPH2* (p.Trp179Leu), and *RP1* (p.Arg1443Gln). All of them were potentially pathogenic variants not previously reported in mutation or polymorphism databases, cosegregating with the disease in the family, predicted as pathogenic with in silico tools and excluded in at least 300 Spanish control chromosomes.

In addition, we found potentially deleterious mutations in other genes with a different model of inheritance. Two known compound heterozygous mutations (p.Arg1129Leu) and (p.Val2050Leu) in the *ABCA4* gene were detected in one family, and two novel mutations in the chromosome X genes, *RPGR* (p.Arg412*) and *RP2* (p.Phe4del). Those were also detected allowing us a genetic and/or even clinical reclassification of three families.

In brief, 16 families presented 17 different variants causative of the disease giving us a mutation detection rate of 27% (16/59) with our strategy. Additionally we could reclassify 5% (3/59) of cases. Those variants were known mutations in 36% (5/17) and the remaining 64% (12/17) were novel mutations.

We have found mutations in 9 of 23 adRP genes included in the RD_NGS_Panel. The frequency of RD causative genes detected in our cohort is represented in Figure 3. The most frequently mutated genes among our cohort were *RP1* found in 7% of families and *PRPH2* and *PRPF31* genes representing 3% of families each.

VUS and Variants Probably not Causative of Disease

Remarkably, 12% (7/59) of cases carried seven unreported nonsynonymous variants in *BEST1*, *FSCN2*, *GUCA1B*, *IMPDH1*, *PRPF8*, and two in *SNRNP200* (Table 2). Although all of them were predicted as very likely pathogenic and they were not present in our Spanish control population, unfortunately cosegregation analysis could not be performed or it was inconclusive to establish the causal relation in the family. Accordingly, they were classified as VUS (Supplementary Fig. S2).

Finally, seven nonsynonymous variants, detected in 10% of studied families (6/59), were classified as variants probably not causative of disease in *IMPG1*, *PRPF3*, *PRPF8*, *PRPH2*, *TOPORS*, and *USH2A* (two variants). Although all changes were predicted as likely pathogenic, variants found in four of these genes were finally excluded as the genetic cause of the disease through cosegregation studies (Supplementary Fig. S2).

In the other two cases, missense changes in*PRPF3* (p.Arg641Gln) and *PRPF8* (p.Met1140Thr) identified in two families, were found in one Spanish chromosome control (MAF: 0.003). Thus, they were considered as rare polymorphic variants in our population.

DISCUSSION

In this study we applied a novel NGS-based strategy to study 59 unrelated index adRP Spanish patients. This is one of the largest cohorts that has been studied by exon capture targeted NGS to date. Our results show 17 mutations detected in 27% of families tested (16/59). From them, 64% (11/17) were first reported here and 36% (6/17) were previously known mutations.

The known mutations detected in this study were not screened before (Supplementary Table S1), except for the known mutations identified in *CRX* and *PRPF31* genes that represented false positives for the ADRP Chip. Additionally, in family RP-0642, SSCP for the *RHO* gene and the genotyping ADRP Chip were performed in the affected member IV:4 instead of the index patient due to its better DNA quality. A new DNA sample of the proband was obtained and NGS was then performed and the mutation (p. Asn15Ser) in the *RHO* gene was found (Supplementary Fig. S1). This known mutation was not detected in IV:4 patient and a subsequent ophthalmic

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TABLE 2. Spectrum of VUS and Variants Probably not Causative of Disease Identified with the RD_NGS_Panel in Autosomal Dominant Retinitis Pigmentosa Families

Family	Gene	NM	HGVS-cdna	HGVS-prot	effect	Zyg	dbSNP (MAF)	SIFT	Polyphen-2	Mutation Taster	Human Splicing Finder	Cosegregation (Affected/ Unaffected/ Asymptomatic)	Frequency in Spanish Control Alleles	Reference
Variant uncertain significance														
RP-1524	*BEST1*	NM_0041833.3	c.1063C>T	p.R355C	Missense	Het	rs139637557 (nfd)	T (0.090)	Pr-D (1.000)	DC (1.000)	-	NA	0/300	-
RP-1684	*PXCN2*	NM_001077182.2	c.445C>T	p.Arg149Trp	Missense	Het	-	D (0)	Pr-D (1.000)	DC (0.999)	-	NA	0/300	-
RP-0992	*GUCA1B*	NM_000322.4	c.119A>G	p.His40Arg	Missense	Het	-	D (0)	Pr-D (1.000)	DC (1.000)	-	In (2/0/1)	0/300	-
RP-1903	*IMPDH1*	NM_000883.3	c.595_597delTTC	p.Phe199del	In-frame	Het	-	-	-	DC (0.999)	-	NA	0/300	-
RP-1187	*PRPH8*	NM_006445.3	c.685T>G	p.Trp229Gly	missense	Het	-	D (0)	Pr-D (1.000)	DC (1.000)	-	In (2/0/1)	0/300	-
RP-1641	*SNRNP200*	NM_0140144	c.1627C>T	p.Pro543Ser	Missense	Het	-	D (0)	Pr-D (1.000)	DC (1.000)	-	NA	0/300	Eisenberger et al.[8] 2013
RP-0723	*SNRNP200*	NM_0140144	c.2579A>G	p.Gln860Arg	Missense	Het	-	D (0.030)	Ps-D (0.870)	DC (1.000)	-	In (2)	0/300	-
Variants probably not causative of disease														
RP-0251	*IMPG1*	NM_001563.2	c.1891G>A	p.Gly631Arg	Missense	Het	-	D (0)	Pr-D (1.000)	DC (1.000)	-	No	0/300	-
RP-0992	*PRPH2*	NM_000322.4	c.299C>T	p.Pro100Leu	Missense	Het	-	D (0.460)	B (0.018)	DC (1.000)	-	No	0/300	-
RP-0258	*PRPF3*	NM_004698.2	c.1922G>A	p.Arg641Gln	Missense	Het	rs201590000 (nfd)	D (0.06)	Pr-D (0.995)	DC (1.000)	-	Yes (3/1)	1/300	-
RP-1649	*PRPH8*	NM_006445.3	c.3419T>C	p.Met1140Thr	Missense	Het	-	D (0)	Pr-D (1.000)	DC (0.998)	-	NA	1/300	-
RP-1608	TOPORS	NM_005802	c.1730C>A	p.Ser577Tyr	Missense	Het	rs79708790 (0.001)	D (0.010)	Ps-D (0.910)	DC (0.981)	-	No	0/300	-
RP-1402	*USH2A*	NM_206933.2	c.1000C>T	p.Arg334Trp	Missense	Het	-	D (0.010)	Pr-D (1.000)	DC (1.000)	-	No	0/150	Adato et al.,[34] 2000
			c.2276G>T	p.Cys759Phe	Missense	Het	rs80338902 (<0.001)	D (0)	Pr-D (1.000)	DC (1.000)	-	No	0/150	Rivolta et al.,[35] 2000

Zyg, zygosity; Het, heterozygosis; nfd, no frequency data; SIFT: deleterious (D) and tolerated (T); Polyphen2: probably damaging (Pr-D), possibly damaging (Ps-D), and benign (B); Mutation Taster: disease causing (DC); P, polymorphism; NA, not available; in, inconclusive.

re-evaluation showed that this patient suffered from congenital stationary night blindness.

All candidate variants detected by NGS were confirmed by Sanger sequencing, therefore no false positives were found in this study. Moreover, all known variants used as positive controls were redetected by this technology. Both results show a high specificity and sensitivity that supports the reliability of our approach.

Due to its novelty, few reports have used NGS approaches to study adRP patients in the last 3 years. These works show a high variability of mutation detection between 23% and 86%.[5–11] The comparison is not straightforward because of differences in cohort size, studies previously made in patients, number of target genes, coverage rates, and other technical issues. Considering similar reports using cohorts of patients previously screened for mutations in the most prevalent genes, the detection mutation rates were similar to our results, approximately 25%[5,10] (Supplementary Table S5). These results probably underestimate the detection rate that these strategies can reach if naïve adRP cases are studied. Moreover, the detection of mutations in less frequent adRP genes was higher than expected for a first screening procedure (Fig. 3).

The inclusion of 73 genes related to RP and LCA independent of the model of inheritance to our NGS approach has had the benefit of allowing us the reorientation of the initial autosomal dominant classification in several families. In two of them, index patients were affected females carrying novel mutations in chromosome X whose pedigrees did not present male-to-male transmission. These findings are not surprising because mutations in the two X-linked RP genes (*RPGR* and *RP2*) have been described previously in 8.5% of families with provisional diagnosis of adRP with affected carrier females.[36] The third family was finally diagnosed as cone–rod dystrophy after detecting known compound heterozygous mutations in *ABCA4*, showing more a pseudodominant pattern of inheritance than a true dominant transmission.

The application of high-throughput sequencing approaches in Mendelian disorders has displayed several limitations particularly in the heterozygous context of dominant forms, as previously described.[37] First, it is not easy to differentiate between true heterozygous variants and false positives of the sequencing. Our NGS approach was designed to obtain high coverage in order to minimize sequencing mistakes described for resequencing technologies. Also, all exons of genes related to adRP included in the panel showed good sequencing depth. Secondly, to distinguish among rare pathogenic and rare benign variants, we have applied a very restrictive pipeline adapted to dominant pathology filtering variants with a MAF less than or equal to 0.001, as recommended previously[37] because all reported adRP mutations are very rare. Additionally, all candidate variants were specifically searched in population-matched controls. We consider that this step is critical to identify rare polymorphic variants that could not be reflected in 1000 genomes and EVS projects. Hence, in our study, all candidate variants detected at least once in our Spanish control cohort were considered as variants probably not causative of disease based on a large MAF. Additionally, cosegregation testing to evaluate the implication of variants is an important tool for dominant Mendelian diseases and sometimes DNA and phenotype information on appropriate family members are unavailable or inconclusive. In those cases, we consider such variants as VUS.

This is the case of the novel missense change (p.Gln860Arg) in *SNRNP200* detected in family RP-0723 that could only be checked in two affected siblings. Moreover, the interpretation of cosegregation results can be also complicated in adRP families with suspected incomplete penetrance. Thus, for the family RP-0992 with the variant p.His40Arg found in *GUCA1B* gene, one mutated member is asymptomatic at 31 years of age, while another affected in the same family showed first symptoms at 12 years of age. Although ophthalmologic follow-up is needed, the occurrence of incomplete penetrance associated to *GUCA1B* mutations has been described previously,[38] therefore we cannot completely exclude that this mutation can be causative of RP in this family.

Cosegregation analysis assuming an autosomal dominant model with complete penetrance, zero recombination fraction, and no phenocopies employed in this work, is equivalent to linkage analysis. Using two-point linkage analysis with more complex trait models could help resolve the probability of causality of variants with possible incomplete penetrance, at least in moderately large families. Although this event has been previously associated with *PRPF8*,[39] the variant p.Trp2279Gly detected in RP-1187 family should be studied in other members of the family to establish the true implication of this change in the disease.

Lastly, the nonsynonymous variant in *PRPH2* (p.Pro100Leu) that putatively induced a critical structural change in the cytoplasmatic domain, was not detected in the affected mother of family RP-0992. However, it was not possible to discard a "de novo" event with two different molecular causes of the disease in this family.

Finally, the information of some variants and mutations in public databases could be incomplete or even misinterpreted.[40] For example the change p.Gln860Arg in *SNRNP200* reported in HGMD Professional database (available in the public domain at www.biobase-international.com/product/hgmd) as mutation, originally was published as VUS in heterozygosis in one case with autosomal recessive LCA.[8] Accordingly, we considered also this variant detected in the index patient of RP-1641 adRP family as VUS. Therefore, the pathogenicity of all variants described previously must be confirmed in original articles.

There are some restrictions in our NGS-based design that could explain the absence of detection of causal mutations in the remaining studied patients. First, novel deep intronic mutations would be missed out because these regions were not included in the panel with the aim to do a cost-effective approach. In addition, some families could carry mutations in *PRPF4* and *HK1*, two genes recently identified associated with adRP and not included in our design.[41,42] Further, up to 2.5% of adRP mutations could be due to genomic rearrangements or deletions in *PRPF31* that could also explain a minor percentage of adRP cases, as previously reported.[43] However, they were not detectable with our bioinformatic analysis. Finally, the presence of the locus RP63 that has been mapped by linkage analysis but for which the causal gene has not yet been identified, suggests that mutations in other genes remain to be discovered.[44]

This work shows that our NGS panel is an effective approach to detect mutations in families affected by adRP and is a feasible tool for application in clinical practice in order to offer a correct diagnosis and genetic counselling. Additionally, our results provide relevant information for mutations and variants found in a large cohort of adRP Spanish patients that increase the knowledge about molecular findings related to RD.

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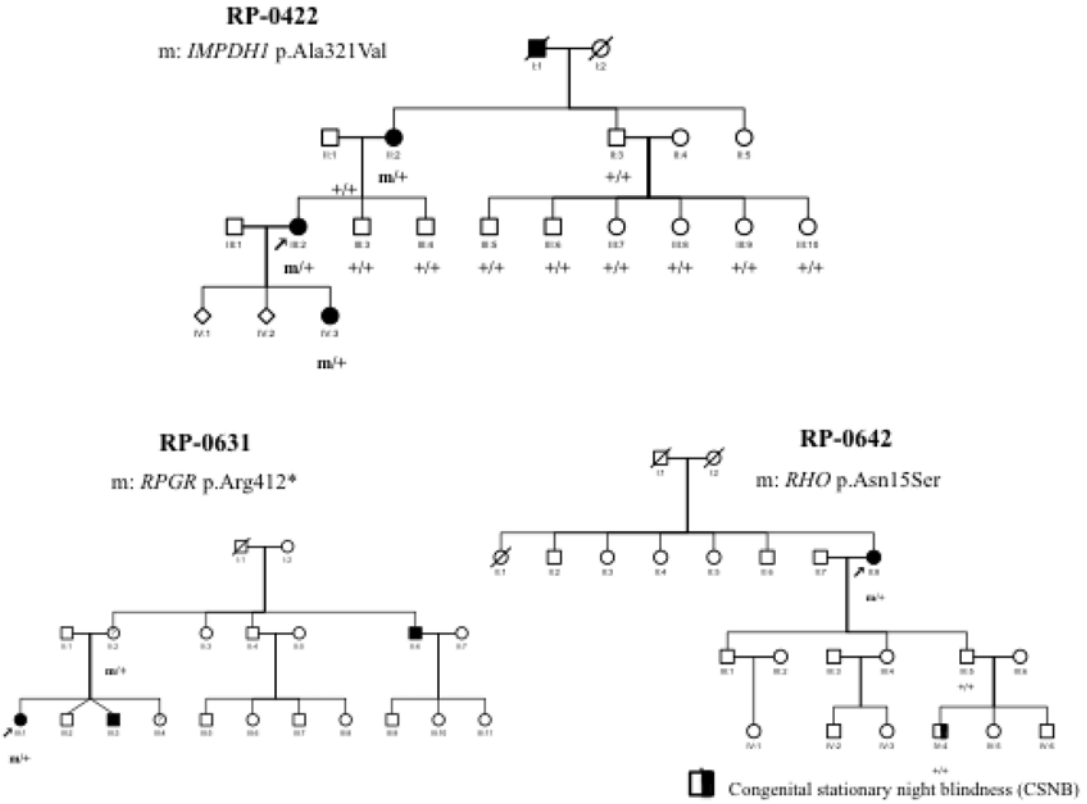
Supporting information

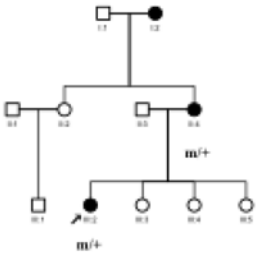
Supplementary Figure 1: Pedigrees of adRP families with mutations causative of the disease detected with theRD_NGS_Panel and co-segregation in available family members. Legend: m/+: mutation detected in heterozygosis; +/+: wild type.

*Incompletely characterized case. Additional molecular studies are in progress to detect another mutated gene involved in the disease.

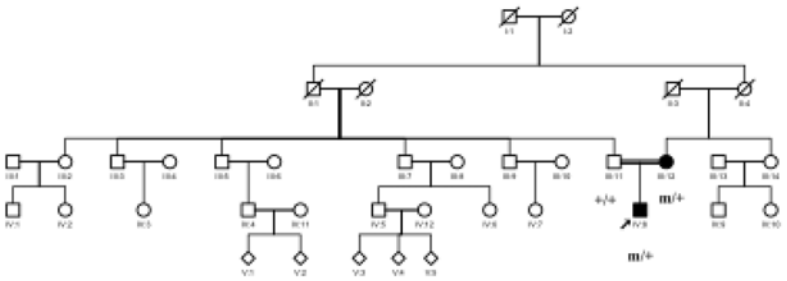
Variants causative of the disease



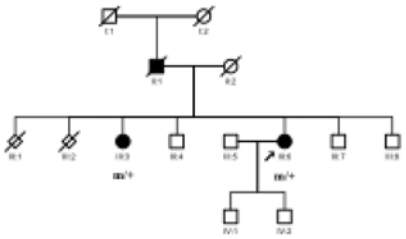
RP-0652
m: *PRPH2* p.Trp179Leu



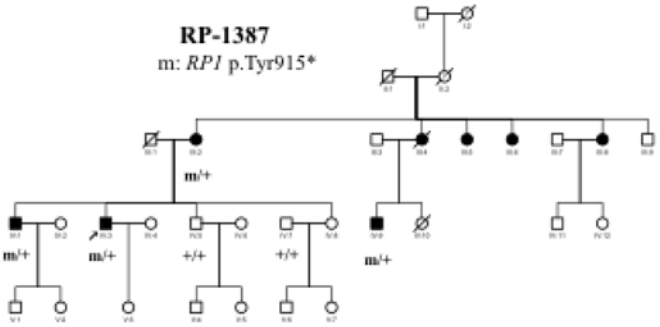
RP-0948
m: *RP1* p.Arg1443Gln



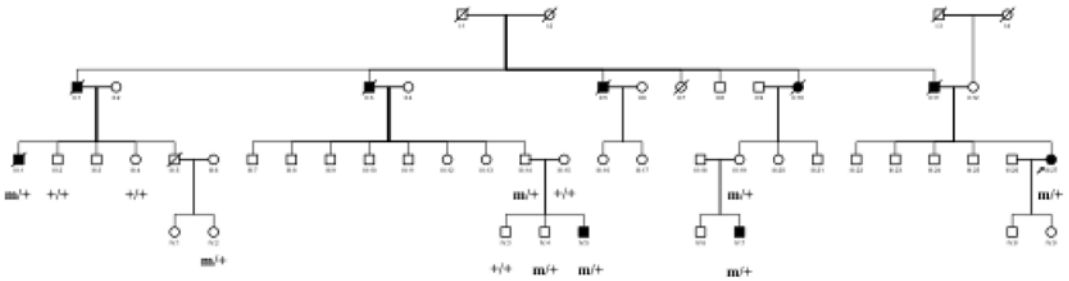
RP-1176
m: *PRPH2* c.582-1G>A



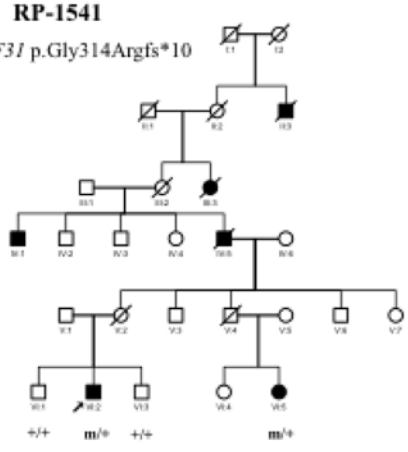
RP-1387
m: *RP1* p.Tyr915*



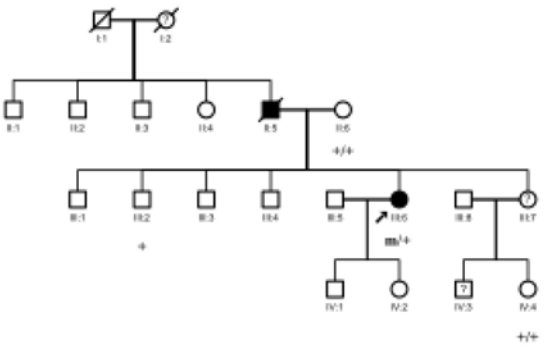
RP-1480
m: *SNRNP200* p.Ser1087Leu



RP-1541
m: *PRPF31* p.Gly314Argfs*10

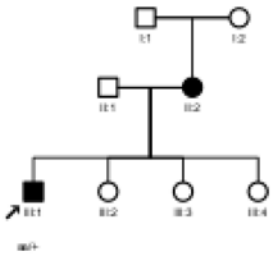


RP-1682
m: *RP2* p. p.Phe4del



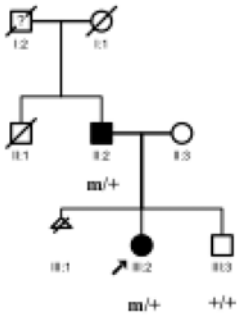
RP-1688

m: *CRX* p.Ala198Glyfs*38



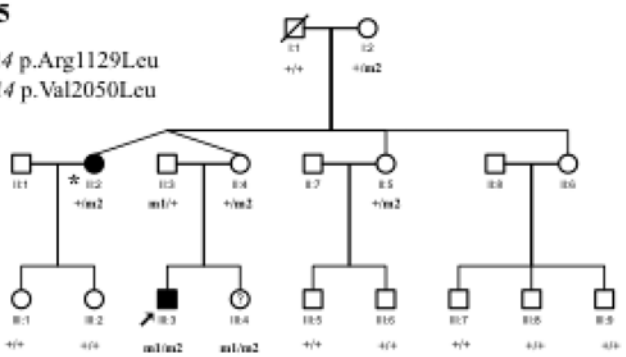
RP-1728

m: *GUCA1B* p.Arg44His



RP-1875

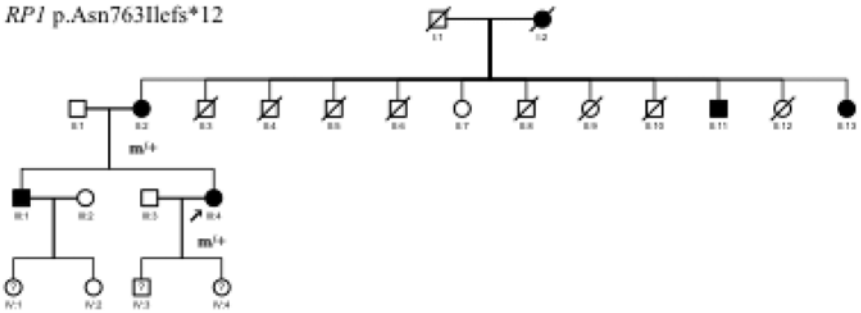
m1: *ABCA4* p.Arg1129Leu
m2: *ABCA4* p.Val2050Leu



*Incompletely characterized case.
Additional molecular studies are in progress to detect another mutated gene involved in the disease.

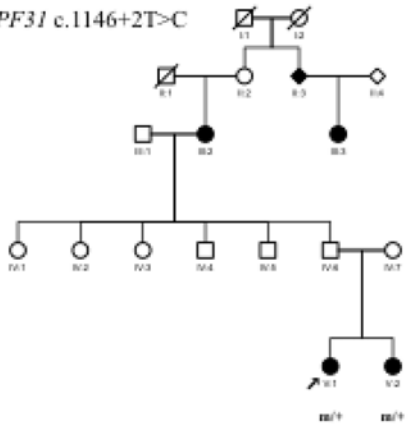
RP-1890

m: *RP1* p.Asn763Ilefs*12



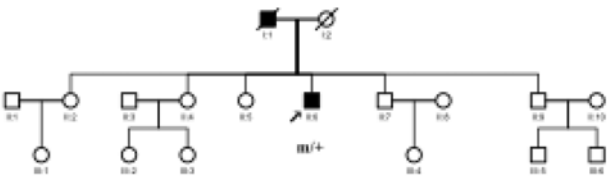
RP-1970

m: *PRPF31* c.1146+2T>C



RP-2072

m: *RP1* p.Glu661*

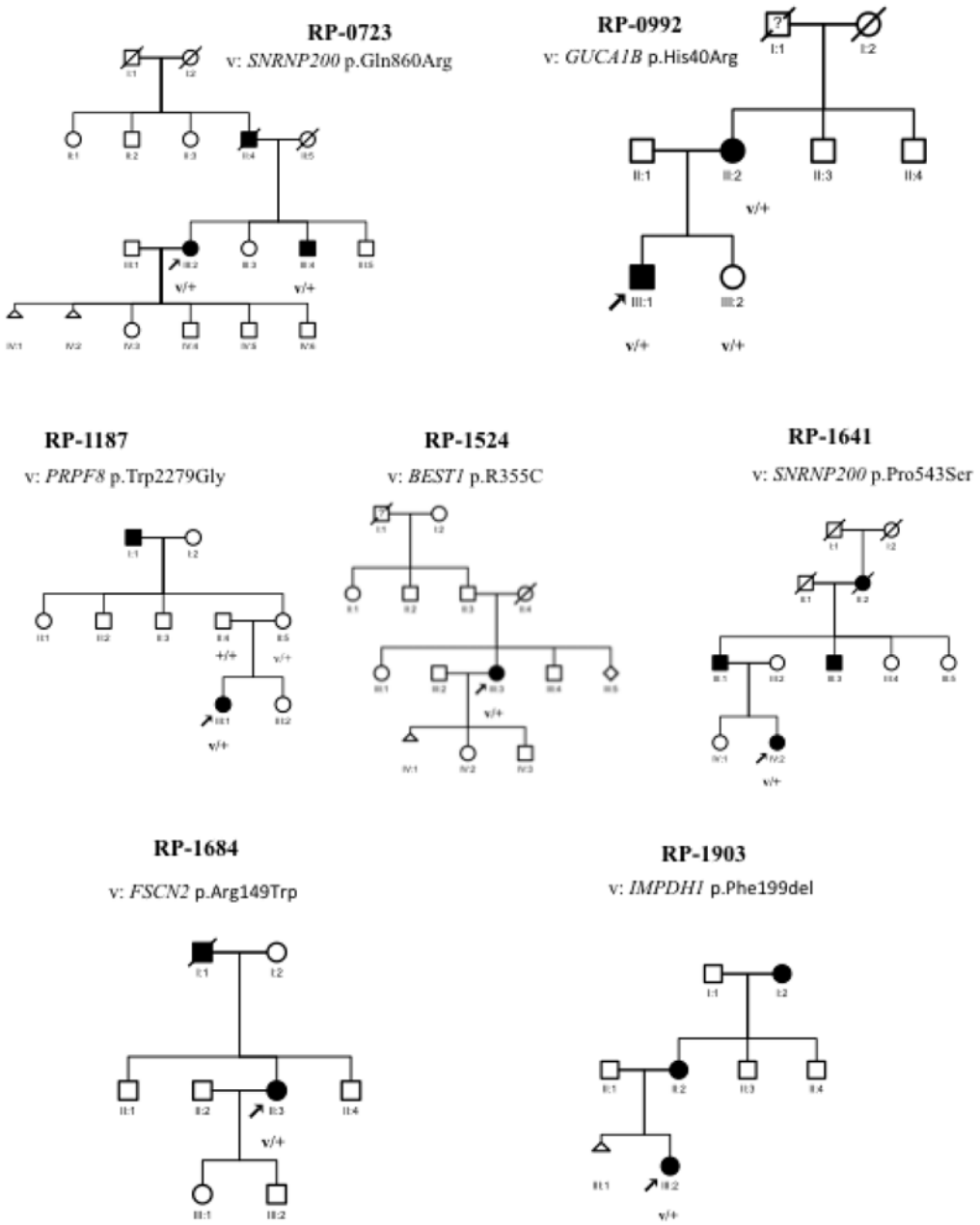


Supplementary Figure 2: Pedigrees of adRP families with variants with uncertain significance (VUS) and variants probably not causative of disease detected with the RD_NGS_Panel and co-segregation analysis in available family members. Legend: v/+: variant detected in heterozygosis; +/+: wild type.

* Variant was not co-segregated with the disease.

**Variant was presented in Spanish population studies.

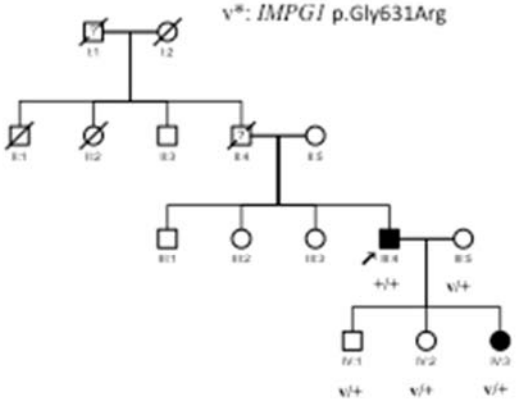
Variants with ucertain significance (VUS)



Variants probably not causative of disease

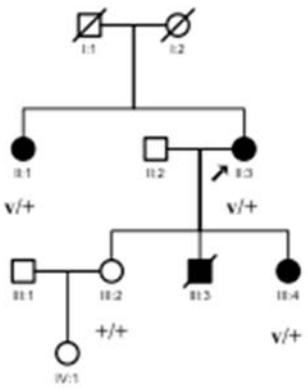
RP-0251

v*: *IMPG1* p.Gly631Arg



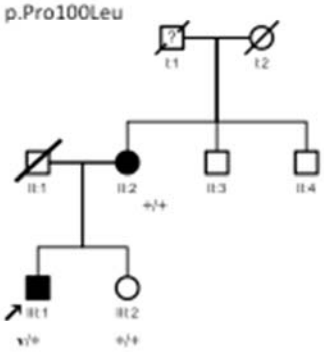
RP-0258

v**: *PRPF3* p.Arg641Gln



RP-0992

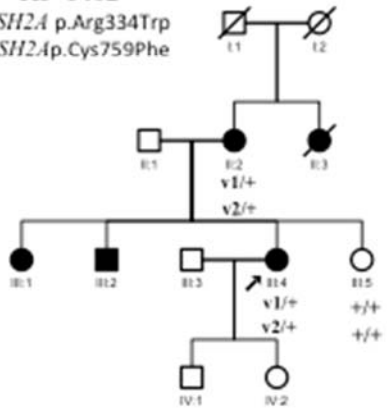
v*: *PRPH2* p.Pro100Leu



RP-1402

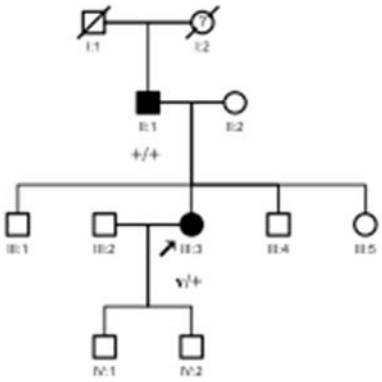
v1*: *USH2A* p.Arg334Trp

v2*: *USH2A*p.Cys759Phe



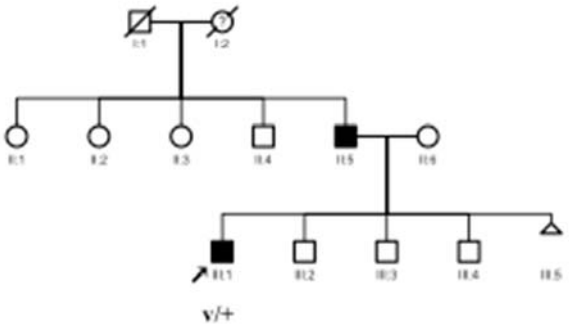
RP-1608

v*: *TOPORS* p.Ser577Tyr



RP-1649

v**: *PRPF8* p.Met1140Thr



Supplementary Table S1: Molecular techniques previously applied in 59 adRP patients.

Techniques		Number of studied families	References
ADRP Chip®		59*	Blanco-Kelly *et al*. 2012[13]
DGGE/SSCP	*CA4*	14	
	CRX	13	
	ELOV4	10	
	FSCN2	11	
	NRL	8	
	PRPF3	16	
	PRPF8	17	
	PRPF31	15	
	PRPH2	14	
	RHO	16*	
	ROM1	9	
	RP1	17	
IMPG1 Sequencing		18	Manes *et al*. 2013[15]
PRPF4 Sequencing		26	Benaglio*et al*. 2014[14]
RHO Sequencing		44	Fernandez-San Jose *et al*. 2014[16]
SNRNP200 (exon 16 and 25) Sequencing		50	

*In family RP-0642 the patient IV:4 was screened instead of index patient due to its better DNA quality.

Supplementary Table S2: Genes associated with RP and LCA included in the customized RD_NGS_Panel.

Gene	cDNA sequence	Number of targets	% Coverage of design	Category	Inheritance	Other described phenotype
ABCA4	NM_000350.2	50	100%	MD	ar	arRP/arCORD
ABHD12	NM_001042472.2	14	100%	RP	ar	RP+ataxia+hearing loss
ADAMTS18	NM_199355.2	23	99.6%	RP	ar	
AIPL1	NM_014336.3	6	95.6%	LCA	ar	adCORD
BBS1	NM_024649.4	17	100%	Ciliopathy	ar	Bardet-Biedl / arRP
BEST1	NM_004183.3	11	100%	MD	ad / ar	arRP / adRP / ad vitreoretinochoroidopathy/ arbestrophinopathy
C2orf71	NM_001029883.2	2	100%	RP	ar	
C8ORF37	NM_177965.3	6	100%	RP	ar	CORD
CA4	NM_000717.3	8	97.0%	RP	ad	
CABP4	NM_145200.3	6	98.3%	CSNB	ar	arCORD / LCA
CEP290	NM_025114.3	55	99.4%	LCA	ar	SLS/ Joubert / MKKS
CERKL	NM_001030311.2	14	98.0%	RP	ar	arCORD with inner retinopathy
CHM	NM_000390.2	16	99.0%	CHM	xl	
CLRN1	NM_174878.2	5	100%	Usher	ar	arRP
CNGA1	NM_001142564.1	12	99.9%	RP	ar	
CNGB1	NM_001297.4	34	99.9%	RP	ar	
CRB1	NM_201253.2	12	100%	LCA	ar	arRP
CRX	NM_000554.4	4	95.5%	LCA	ar / ad	adRP / adCRD
CYP4V2	NM_207352.3	11	97.6%	RP	ar	Bietti crystalline corneoretinal dystrophy
DHDDS	NM_024887.3	9	100%	RP	ar	
EYS	NM_001142800.1	45	99.2%	RP	ar	
FAM161A	NM_032180.2	7	100%	RP	ar	
FSCN2	NM_001077182.2	5	100%	RP	ad	
GUCA1B	NM_000180.3	4	96.2%	RP	ad	
GUCY2D	NM_000180.3	20	98.5%	LCA	ar	adRP / adCRD
IDH3B	NM_006899.3	12	100%	RP	ar	
IMPDH1	NM_000883.3	17	100%	RP	ad	
IMPG1	NM_001563.2	17	100%	RP	ar	
IMPG2	NM_016247.3	19	99.7%	RP	ar	
IQCB1	NM_001023570.2	15	99.8%	LCA	ar	SLS
KCNJ13	NM_002242.4	3	100%	LCA	ar	adVRP
KLHL7	NM_001031710.2	14	100%	RP	ad	
LCA5	NM_181714.3	9	99.9%	LCA	ar	
LRAT	NM_004744.3	3	97.8%	RP	ar	LCA
MAK	NM_001242957.1	13	98.2%	RP	ar	
MERTK	NM_006343.2	19	100%	RP	ar	LCA / arCRD
MPDZ	NM_003829.4	45	100%	RP	ar	
NMNAT1	NM_022787.3	5	93.4%	LCA	ar	

NR2E3	NM_014249.2	8	100%	RP	ar / ad	arESC; Goldmann-Favre syndrome; combined ad and ar retinopathy
NRL	NM_006177.3	3	100%	RP	ar / ad	
OFD1	NM_003611.2	24	100%	XLRP	xl	Joubert
PDE6A	NM_000440.2	22	98.4%	RP	ar	
PDE6B	NM_000283.3	23	99.5%	RP	ar	ad CNSB
PDE6G	NM_002602.3	4	100%	RP	ar	
PRCD	NM_001077620.2	6	100%	CORD	ar	arRP
PROM1	NM_006017.2	29	100%	MD	ad / ar	arRP/ adCRD
PRPF3	NM_004698.2	16	100%	RP	ad	
PRPF31	NM_015629.3	15	97.4%	RP	ad	
PRPF6	NM_012469.3	21	100%	RP	ad	
PRPF8	NM_006445.3	43	99.8%	RP	ad	
PRPH2	NM_000322.4	3	100%	RP	ad	adMD / adCRD / digenic retinitis pigmentosa with ROM1
RBP3	NM_002900.2	4	100%	RP	ar	
RD3	NM_183059.2	3	100%	LCA	ar	
RDH12	NM_152443.2	9	100%	LCA	ar / ad	arRP/adRP
RGR	NM_001012720.1	7	91.0%	RP	ar	ad choroidal sclerosis
RHO	NM_000539.3	5	100%	RP	ad / ar	ad CNSB
RLBP1	NM_000326.4	9	100%	RP	ar	arCORD / ar Bothnia dystrophy / ar retinitis punctataalbescens
ROM1	NM_000327.3	3	100%	RP	ad	digenic retinitis pigmentosa with PRPH2
RP1	NM_006269.1	4	100%	RP	ad / ar	
RP2	NM_006915.2	5	98.4%	RP	xl	
RP9	NM_203288.1	6	84.6%	RP	ad	
RPE65	NM_000329.2	14	98.0%	LCA	ar	arRP
RPGR	NM_001034853.1	15	84.8%	RP	xl	xlCRD / xlMD
RPGRIP1	NM_020366.3	24	100%	LCA	ar	arCRD
SAG	NM_000541.4	16	99.6%	RP	ar	CNSB / arOguchi disease
SEMA4A	NM_022367.3	16	100%	RP	ad	adCRD
SNRNP200	NM_014014.4	45	99.6%	RP	ad	
SPATA7	NM_018418.4	12	98.9%	LCA	ar	arRP
TOPORS	NM_005802.4	3	100%	RP	ad	
TTC8	NM_144596.2	15	100%	RP	ar	Bardet-Biedl syndrome
TULP1	NM_003322.3	15	100%	RP	ar	LCA
USH2A	NM_206933.2	73	100%	RP	ar	Usher
ZNF513	NM_144631.5	4	100%	RP	ar	

Legends: macular dystrophy (MD); retinitis pigmentosa (RP); Leber'scongenital amaurosis (LCA); congenitalstationarynightblindness (CSNB); choroideremia (CHM), cone-roddystrophy (CRD); autosomal recessive (ar); autosomal dominant (ad); X-linked (xl); McKusick-KaufmanSyndrome (MKKS); SeniorLokenSyndrome (SLS); vitreoretinopathyproliferative (VRP); enhanced S-conesyndrome (ESC).

Supplementary Table S3: Deep intronic mutations included in the RD_NGS_Panel.

Gene	RefSeq Isoform	HGVS-cDNA	HGVS-prot	effect
USH2A	NM_206933.2	c.7595-2144A>G	Lys2532Thrfs*56	intron
CEP290	NM_025114.3	c.2991+1655A>G	p.C998*	intron
PRPF31	NM_015629.3	c.1374+654C>G	-	intron
OFD1	NM_003611.2	c.935+706A>G	-	intron

Supplementary table 4: Families with previously identified variants used as positive controls to evaluate the NGS approach

Family	Gene	HGVS-cDNA	HGVS-prot	Effect
RP-0377	***RHO***	c.-26A>G		5' UTR
		c.696+4C>T		intron
		c.*43C>A		3' UTR
RP-0431	***RHO***	c.-26A>G		5' UTR
		c.696+4C>T		intron
		c.*43C>A		3' UTR
RP-0932	***RHO***	c.*43C>A		3' UTR
RP-1247	***RHO***	c.696+4C>T		intron
RP-1252	***IMPG1***	c.151dupA	p.Met51Asnfs29	frameshift
RP-1371	***RP1***	c.1118C>T	p.Thr373Ile	missense
RP-1402	***RHO***	c.696+4C>T		intron
		c.*43C>A		3' UTR
RP-1480	***RHO***	c.-26A>G		5' UTR
		c.696+4C>T		intron
		c.*43C>A		3' UTR
	FSCN2	c.967G>A	p.Ala323Thr	missense
RP-1552	***RHO***	c.-26A>G		5' UTR
		c.696+4C>T		intron
		c.*43C>A		3' UTR
RP-1587	***RHO***	c.-51G>A		5' UTR
		c.-26A>G		5' UTR
RP-1616	***RHO***	c.696+4C>T		intron
RP-1649	***USH2A***	c.2276G>T	p.Cys759Phe	missense
RP-1728	***SNRNP200***	c.3315A>G	p.Ala2005Ala	synonymous
RP-1794	***RHO***	c.-26A>G		5' UTR
		c.696+4C>T		intron

		c.*43C>A	3' UTR
RP-1801	***RHO***	c.-26A>G	5' UTR
RP-1815	***RHO***	c.-26A>G	5' UTR

Study	Cohort of adRP families*	Cases previously studied	Detection rate for adRP	Enrichment/Platform	Nº genes studied
Bowne *et al.* 2011[5]	21	yes	24 % (5/21)	PCR/ GS FLX (Roche)-GAIIx (Illumina)	46
Audo*et al.*2012[6]	17	some	57 % (10/17)	SureSelect/ GAIIx (Illumina)	254
O´Sullivan *et al.*2012[7]	14	yes	64 % (9/14)	SureSelect/ SOLiD 4 (Life Tech)	50
Eisenberg *et al.*2013[8]	14	NA	86 % (12/14)	Nimblegen/ GS FLX (Roche)	55
Glöckle*et al.*2013[9]	29	NA	41 % (12/29)	SureSelect/ SOLiD 4 (Life Tech)	105
Daiger*et al.*2014[10]	21	yes	24 % (5/21)	PCR/GAIIx (Illumina)	46
	13	yes	23 % (3/13)	SureSelect/Illumina platform	172
Oishi*et al.*2014[11]	77	some	35 % (27/77)	Haloplex/ HiSeq2500 (Illumina)	365
This study	59	yes	31% (18/59)	Haloplex/ GAIIx (Illumina)	73

Supplementary Table S5: Summary of next generation sequencing approaches applied in autosomal dominant Retinitis Pigmentosa families.

CAPÍTULO V

Identificación de nuevos genes asociados a retinosis pigmentaria mediante secuenciación de exoma completo

Contexto

La utilización de herramientas de secuenciación masiva para el diagnóstico genético ha revolucionado completamente el campo de la genética humana. Así, con la mejora en la eficiencia, el coste de la secuenciación y el tiempo de estudio, la mejora en los sistemas informáticos así como el aumento en el conocimiento de las variantes, la secuenciación de exoma completo y genoma completo se han postulado como buenas estrategias para la identificación de nuevos genes asociados a patologías con componente hereditario. De este modo, un total de 53 genes nuevos relacionados con DR han sido identificados mediante estas tecnologías. Se estima que aproximadamente el 50% de los casos de adRP presentan mutaciones en genes nuevos no asociados a esta patología. En estos casos la técnica *WES*, dirigida a toda la región codificante del genoma, podría ser la herramienta adecuada para ayudar a detectar los genes causantes de adRP que quedan aún por identificar.

Nuestro grupo tuvo la oportunidad de adquirir experiencia en el manejo de la tecnología de *WES*, en colaboración con el grupo de genómica del *Children´s Hospital of Philadelphia* y el Instituto Genómica de Pekín (*Beijing GenomicsInstitute, BGI*), aplicando dicha herramienta a un grupo de siete familias con adRP reclutadas en nuestro laboratorio. Para ello, se seleccionaron familias altamente informativas entre nuestra cohorte de 200 familias con disponibilidad de individuos afectos y no afectos para posteriores estudios de segregación,que hubieran sido ampliamente cribadas para genes y mutaciones conocidas asociados a adRP mediante técnicas moleculares convencionales, con el fin de tratar de identificar nuevos genes asociados a RP. Como resultado de este trabajo, se publicaron los siguientes dos artículos.

Artículo*: Expanding the phenotype of PRPS1 syndromes in females: neuropathy, hearing loss and retinopathy.*

En este trabajo se utilizó la secuenciación de exoma completo para la caracterización molecular de una familia con aparente herencia autosomica dominante, identificándose una mutación nueva, p.Ser16Pro, en el gen *PRPS1*, ligado al cromosoma X, en una familia cuyas mujeres portadoras presentaban distintos grados de RP de inicio y progresión muy variables, además de distintos grados de atrofia óptica, polineuropatía e hipoacusia.

Este gen había sido previamente asociado a otras patologías como el Sídrome de Arts (MIM 301835), el síndrome de Charcot-Marie-Tooth tipo 5 Ligado al cromosoma X (CMTX5, MIM 311070); la sordera neurosensorial no sindrómica ligada al cromosoma X (DFN2, MIM 304500) y el síndrome de Superactividad de PRS-I (MIM 300661), todos ellos asociados a distintas manifestaciones clínicas que en ningún caso incluían síntomas de DR, por lo que este hallazgo nos permitió establecer una nueva asociación fenotípica para este gen.

Con el fin de identificar un nuevo gen asociado a formas dominantes sindrómicas, se obtuvo la secuenciación exómica completa en las tres mujeres afectas de esta familia, obteniendo un promedio de 48.306 variantes tipo S*NVs* y 8.218 inserciones/deleciones (*indels)* que fueron filtradas considerando su localización en en región codificante o zonas de *splicing* canónicas, altamente conservadas evolutivamente y que se predijeran como patogénicas en al menos dos de los cuatro sistemas *in silico* que fueron utilizados. Además se tuvieron en cuenta datos de frecuencias poblacionales, su descripción previa de en bases de datos y literatura, además de considerar la relevancia biológica y clínica de acuerdo a la función y la expresión génica. De este modo se seleccionaron 12 variantes candidatas, de las que únicamente segregó completamente en la familia un nuevo cambio *missense* en el exón 1 de *PRPS1*, c.46 T> C, p.Ser16Pro (NM_001204402), que codificaba para la enzima PRS-I.

Para evaluar el efecto funcional de la mutación *in vitro*, se determinó la actividad enzimática del PRS-I en eritrocitos de las tres mujeres afectadas donde se evidenciaron diferentes niveles de deficiencia enzimática. Mediante análisis de expresión en ARN procedente de linfocitos de sangre periférica, se descartaron posibles alteraciones en el patrón de reconocimiento de *splicing* de ARNm que habían sido sugeridas mediante análisis predictivos. Sin embargo, se identificó una expresión alelo-específica, estando ausente el alelo "silvestre", en linfocitos del caso índice de la familia, causada por un sesgo significativo en la

inactivación del cromosoma X "silvestre" en esta muestra. Finalmente se pudo determinar una correlación entre la inactivación del cromosoma X en leucocitos y el grado de deficiencia de la enzima PRS-I en los eritrocitos con la gravedad del fenotipo.

El estudio de esta familia mediante exoma completo nos ha permitido asociar por primera vez dicho gen a mujeres con distrofia de retina, establecer una nueva asociación y ampliar así el espectro de fenotipos asociados a *PRPS1.* Los resultados presentados en este trabajo apoyaron los hallazgos previos que evidenciaban la existencia de fenotipos intermedios en los déficit de enzima PRS-I y han demostrado que las mujeres portadoras de las mutaciones de *PRPS1* pueden encontrarse tan severamente afectadas como sus homólogos masculinos y, por lo tanto, este síndrome debe ser también considerado en mujeres con RP sindrómica incluso en ausencia de varones afectados en la familia. La caracterización genética de las familias es de vital importancia para de poder proporcionar un adecuado pronóstico de la evolución clínica de la enfermedad y asesoramiento genético a las familias afectadas.

Aportación de la autora

En este trabajo, la autora estuvo implicada en la recopilación de datos clínicos y genéticos y de muestras de cada uno de los miembros de la familia estudiada, en la validación y segregación mediante secuenciación Sanger de las 12 variantes candidatas identificadas en cuatro miembros afectos y ocho miembros sanos de la familia. Además, participó en el filtrado, análisis e interpretación de resultados obtenidos mediante la tecnología *WES* aplicada, en el análisis *in silico* de patogenicidad de la mutación p.Ser16Pro, estudio de su conservación evolutiva, el impacto de la mutación en la estructura tridimensional de la proteína y la predicción en la alteración del *splicing* del mRNA. También realizó el ensayo de inactivación del cromosoma X y estuvo implicada en la interpretación de resultados tanto del análisis de expresión del gen, como la determinación de la actividad enzimática en eritrocitos. Además participó en el análisis de correlación fenotipo-genotipo y en la revisión de la literatura relacionada con la descripción de todos los fenotipos asociados a la alteración del gen *PRPS1*. Por último, la doctoranda participó en la escritura del manuscrito que se presenta en este trabajo.

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RESEARCH Open Access

Expanding the phenotype of *PRPS1* syndromes in females: neuropathy, hearing loss and retinopathy

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Abstract

Background: Phosphoribosyl pyrophosphate synthetase (PRS) I deficiency is a rare medical condition caused by missense mutations in *PRPS1* that lead to three different phenotypes: Arts Syndrome (MIM 301835), X-linked Charcot-Marie-Tooth (CMTX5, MIM 311070) or X-linked non-syndromic sensorineural deafness (DFN2, MIM 304500). All three are X-linked recessively inherited and males affected display variable degree of central and peripheral neuropathy. We applied whole exome sequencing to a three-generation family with optic atrophy followed by retinitis pigmentosa (RP) in all three cases, and ataxia, progressive peripheral neuropathy and hearing loss with variable presentation.

Methods: Whole exome sequencing was performed in two affecteds and one unaffected member of the family. Sanger sequencing was used to validate and segregate the 12 candidate mutations in the family and to confirm the absence of the novel variant in *PRPS1* in 191 controls. The pathogenic role of the novel mutation in *PRPS1* was assessed *in silico* and confirmed by enzymatic determination of PRS activity, mRNA expression and sequencing, and X-chromosome inactivation.

Results: A novel missense mutation was identified in *PRPS1* in the affected females. Age of onset, presentation and severity of the phenotype are highly variable in the family: both the proband and her mother have neurological and ophthalmological symptoms, whereas the phenotype of the affected sister is milder and currently confined to the eye. Moreover, only the proband displayed a complete lack of expression of the wild type allele in leukocytes that seems to correlate with the degree of PRS deficiency and the severity of the phenotype. Interestingly, optic atrophy and RP are the only common manifestations to all three females and the only phenotype correlating with the degree of enzyme deficiency.

Conclusions: These results are in line with recent evidence of the existence of intermediate phenotypes in PRS-I deficiency syndromes and demonstrate that females can exhibit a disease phenotype as severe and complex as their male counterparts.

Keywords: *PRPS1*, Retinitis pigmentosa, Non-random X-chromosome inactivation, Phosphoribosyl pyrophosphate synthetase deficiency, Neuropathy

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Background

Phosphoribosyl pyrophosphate synthetase 1 (PRS-I, [MIM 311850]) is an ubiquitous enzyme with an essential role in nucleotide metabolism: it catalyzes the synthesis of phosphoribosyl pyrophosphate (PRPP), the substrate for the synthesis of purine, pyridine, and pyrimidine nucleotides [1]. PRS-I has two highly homologous isoforms, PRS-II (MIM 311860) widely expressed as PRS-I, and PRS-III (MIM 611566) whose expression is restricted to testes. The three isoforms are products of separate but highly conserved genes: *PRPS1* (Xq22.3), *PRPS2* (Xp22.2), and *PRPS1L1* (7p21.1), respectively [2]. Missense mutations in *PRPS1* are rare and may result in increase or decrease in PRS-I activity. PRS-I deficiency is an extremely rare condition that can lead to three different disorders: Arts syndrome (MIM 301835), being the most extreme form of enzyme deficiency; Rosenberg-Chutorian syndrome or X-linked Charcot-Marie Tooth 5 (CMTX5, MIM 311070), which represents a less severe phenotype; and X-linked non-syndromic sensorineural deafness (DFN2, MIM 304500), as the mildest form of deficiency. Only twelve families have been described so far with PRS-I deficiency syndromes [3-7] [I. del Castillo, personal communication]. Hearing loss is the only common feature between the three disorders and the only symptom observed in DFN2. Arts syndrome and CMTX5 share additional neurological anomalies such as ataxia, hypotonia, and optic atrophy [4,8,9]. Arts syndrome is also characterized by delayed motor development, mild to moderate intellectual disability and frequent recurrent infections that typically result in early death [3,10]. PRS-I superactivity (MIM 300661) is more frequent than the deficiency and is characterized by hyperuricemia and gout and it can be accompanied by other neurological symptoms such as sensorineural deafness, hypotonia, mental retardation, and also by recurrent infections [11,12]. All four disorders are inherited in a recessive X-linked manner so only males are affected. Obligate female carriers, however, may occasionally display milder symptoms such as hearing loss in CMTX5 [8,9], hearing impairment, ataxia, hypotonia or hyperreflexia in Arts syndrome [3], or hyperuricemia in PRS-I superactivity [13].

Using whole exome sequencing (WES), we identified a novel loss-of-function mutation in *PRPS1* leading to enzyme deficiency in three females with optic atrophy (OA), retinitis pigmentosa (RP), ataxia, peripheral neuropathy and hearing loss with variable presentation.

Methods

Subjects

A three-generation Spanish family (RP-0482) consisting of four affected females (Figure 1A) was recruited and evaluated by the Fundacion Jimenez Diaz Hospital (Madrid, Spain). The four affecteds, six unaffecteds and two unrelated members of the family participated in the genetic study. All four affected females, II:2, III:2, IV:2 and IV:3, displayed typical or sectorial RP and various degrees of neurological symptoms. II: 2 and II:3 died during the course of the study but we obtained their DNA samples and informed consent before allowing us to include them in the study. Additionally, 191 unrelated Spanish individuals with no history of retinal dystrophy and randomly selected from blood donors voluntarily participated as controls. Informed consent was obtained from all individuals involved, all procedures were reviewed and approved by the Ethics Committee of the Hospital and adhered to the tenets of the Declaration of Helsinki.

Whole exome sequencing and variant analysis

Genomic DNA was captured by Agilent SureSelect Human All Exon kit version 2 covering 46 MB of coding region (Agilent Technologies, Santa Clara, CA, USA), and sequenced on HiSeq 2000 instruments (Illumina, San Diego, CA, USA). Raw reads were mapped to the human reference genome (UCSC hg19), using the Burrows–Wheeler alignment tool [15]. Genome Analysis Tool Kit version 1.4 [16] was used for variant calling. ANNOVAR [17] was used for variant functional annotation and prediction and conservation scores from SIFT [18], Polyphen2 [19], LRT [20], MutationTaster [21], PhyloP [22] and GERP++ [23] were retrieved from the Database for Nonsynonymous SNPs' Functional Predictions [24] for every potential nonsynonymous single nucleotide variant (SNVs).

Variant filtering was performed under the assumption of dominant inheritance. Only nonsynonymous, splice site disrupting, and frameshift heterozygous variants segregating in the family with a minor allele frequency ≤0.5% in a control cohort of more than 8000 individuals (1000 Genomes Project, [25] (April, 2012 release); 6503 exomes from NHLBI GO Exome Sequencing Project [26], and 669 in-house whole-exomes) were considered. Further gene prioritization was performed combining data on minor allele frequency, sequence conservation, potential deleterious effect and biological and clinical relevance according to gene function, gene expression, and the existence of mutation reports in databases such as The Human Gene Mutation Database [27], The Retinal Information Network (RetNet) [28] or Online Mendelian Inheritance in Man (OMIM) [29].

Sanger sequencing

Sanger sequencing was used for the segregation of all variants resulting from the filtering analysis in family RP-0482 and to confirm the absence of variant p.Ser16-Pro in *PRPS1* in 191 Spanish controls (primers available in Additional file 1). PCR products were enzymatically purified with ExoSAP-it (USB, Affymetrix), sequenced on both strands using Big Dye Terminator Cycle Sequencing

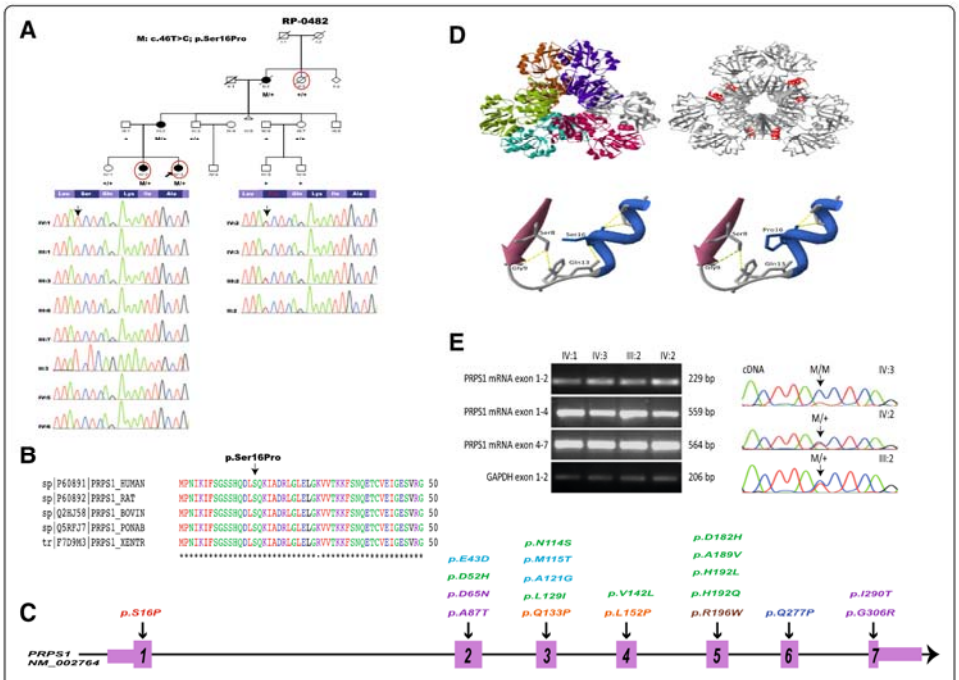

Figure 1 *PSPS1* is mutated in females with syndromic retinitis pigmentosa from a three-generation family. A. Pedigree of family RP-0482 and validation by Sanger sequencing of p.Ser16Pro demonstrating the correct segregation in the family. All affected individuals (II:2, III:2; IV:2 and IV:3) are heterozygous for the variant. Red circles indicate the individuals analysed by WES. Although II:2 and II:3 died during the course of the study, DNA samples and informed consents were obtained before deceasing, allowing us to include those subjects in the segregation analysis. **B.** Multiple sequence alignment of *PRPS1* across species using ClustalW [14] confirms that p.Ser16 is identical from human to zebrafish. **C.** Schematic representation of *PRPS1* with the location of the novel heterozygous mutation p.Ser16Pro in exon 1 (in red) and previously known mutations in Arts syndrome (in orange), PRS-I Superactivity (in green), Charcot-Marie-Tooth disease-5 (in blue), X-linked nonsyndromic sensorineural deafness (in purple), and also the recently reported mutation associated with retinal dystrophy (in brown). Exons are indicated by rectangles that are wider for the coding regions. Nucleotide numbering reflects cDNA in the reference sequence NM_002764. **D.** Model of PRS-I with p.Ser16Pro based on the crystal structure of human PRS-I (PDB: 2H06) and close-up of the mutation showing the loss of a hydrogen bound with residue Gln13. **E.** RT-PCR and sequencing analysis of p.Ser16Pro in mRNA. RNA was derived from peripheral blood lymphocytes of the three patients (IV:2, IV:3 and III:2) and an unaffected control (IV:1). Normally spliced amplicons of exons 1–2, 1–4 and 4–7 of *PRPS1* comprising 229, 559 and 564 nucleotides, respectively, were found in all cases. Amplification of *GAPDH* mRNA analysis was used as positive control. Sanger sequencing of RT-PCR products evidences the absence of the wild-type allele in the cDNA of the proband (IV:3).

Kit v3.1 (Applied Biosystems) and resolved on an automated sequencer (ABI 3130xl Genetic Analyzer, Applied Biosystems).

In silico analyses of p.Ser16Pro pathogenicity

The evolutionary conservation of p.Ser16Pro was assessed by multiple sequence alignment of *PRPS1* across species using ClustalW2 [14]. The impact of the mutation in the tridimensional structure of the protein was assessed with a model of PRS-I with p.Ser16Pro based on the crystal structure of the human protein (PDB: 2H06) using Swiss Model [30,31] and Swiss PDB viewer [32]. ESEFinder [33,34] was used to determine whether p.Ser16Pro could alter the normal splicing of the mRNA.

Determination of PRS activity in erythrocytes

PRS enzymatic activity was determined in erythrocytes from III:2, III:3, IV:1, IV:2, and IV:3, according to the method previously described by Torres et al. [35]. Interval used as reference was 70–126 nmol/h/mg hemoglobin.

PRPS1 expression analysis

PRPS1 expression analysis was performed in RNA samples using blood lymphocytes from III:2, IV:1, IV:2, and

IV:3. Total RNA was reversely transcribed to cDNA with ImProm-II™ Reverse Transcription System (Promega) using random primers. RT-PCR experiments were performed using *PRPS1* exonic primers pairs spanning exons 1–2, 1–4, and 4–7 (primers available in the Additional file 1). Primers for the housekeeping *GAPDH* gene were used as internal control. PCR fragments were subjected to electrophoresis in a gel searching for abnormally spliced amplicons and further sequenced.

X-chromosome inactivation

X chromosome inactivation assay was performed in sodium bisulfite-treated genomic DNA from peripheral blood and saliva (EpiTect Bisulfite Kit, Quiagen) by a methylation specific PCR of the first exon of the human androgen receptor *locus* with fluorochrome-coupled primers. Heterozygosity of the human androgen receptor region in the target samples was previously checked. Two different pairs of primers were used to detect the methylated and unmethylated alleles and PCR fragments were analyzed in an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). X chromosome inactivation skewing was reported as percentage of the predominant allele and was considered skewed when the predominant allele exceeded 74%, non-skewed between 50% and 65% and undetermined between 66% and 74% [36].

Results

Familial history of RP-0482

Data from ophthalmological, neurological and otological examinations of patients III:2, IV:2, and IV:3 were available from a period of more than 15 years and the phenotype is described in detail in the Additional file 1. Patient II:2 also displayed sectorial RP and ataxic traits, but the progression of the clinical phenotype is not available. Figure 2 shows the retinal and MRI images of the three affected females. Age of onset, presentation and severity of the phenotype are highly variable in the family, as summarized in Table 1. Both the index patient and her mother have various degrees of ataxia, peripheral neuropathy and hearing loss beyond the ophthalmological symptoms, whereas the phenotype of the affected sister is currently confined to the eye and milder than those of III:2 and IV:3 (Additional files 1 and 2)

Identification of a novel missense mutation in *PRSP1* by WES

DNA samples from individuals III:3, IV:2, and IV:3 were subjected to WES (Figure 1A). A total of 10.77 GB data on target genomic regions was generated for the three samples, with a mean coverage of target region of 78.23X. An average of 48,306 SNVs and 8,218 insertion/deletion (indels) were called for the three exomes, but for further filtering only coding variants were considered, thus reducing the number to an average of 18,722 SNVs and 741 indels per sample. Variant filtering under the assumption of a dominant inheritance yielded 141 variants in 126 genes as potential candidates (Additional file 3) with no known cause of retinal degeneration amongst them (RetNet, [28] accessed June, 2013). Further filtering of the 141 variants, left 12 novel variants, conserved across species according to the values from PhyloP and GERP++, predicted to be pathogenic at least by two of the systems evaluated and with expression in the retina, so they were selected for validation and segregation in the family (Additional file 1). Only the novel missense change in exon 1 of *PRPS1*, c.46 T > C; p.Ser16Pro (NM_001204402), completely segregated in all four affecteds and eight unaffected members of family RP-0482 (Figure 1A) and was absent in controls (258 X chromosomes total). Both GERP++ and PhyloP values estimated a high degree of conservation of serine at position 16 of PRS-I across species (Additional file 3), and was confirmed by multiple sequence alignment (Figure 1B), being the first missense mutation identified in this gene from the first α-helix to the fourth β-strand (aminoacids 1 to 43, Figure 1C). All *in silico* programs evaluated except for Polyphen2 predicted p.Ser16Pro to be damaging (Additional file 3). The tridimesional model of PRS-I with p.Ser16Pro indicated that Ser16 is located in the first α-helix of the protein in the N-terminal domain (Figure 1D). The replacement of serine with proline leads to the loss of a hydrogen bond with Gln13 likely breaking the tightly packed α-helix.

p.Ser16Pro leads to PRS deficiency in females

To further assess the functional effect of the mutation in vitro, PRS enzymatic activity was determined in erythrocytes from three affected females (III:2, IV:2, and IV:3), where different levels of enzyme deficiency were evidenced (Table 1), and two unaffecteds (III:3 and IV:1) with PRS-I within the normal range.

Lack of expression of *PRPS1* p.Ser16 in the index patient

The *in silico* analysis using ESEFinder predicted this mutation to alter the recognition pattern of splicing RNA proteins compared to the wild type sequence. To confirm this, *PRPS1* expression analysis was performed in RNA samples from blood lymphocytes from three affecteds and one unaffected (IV:1). RT-PCR analysis yielded no differences on the expression of *PRPS1* transcripts between carriers of p.Ser16Pro and the non-carrier (Figure 1E), and no additional splicing transcripts were found. Notably, further sequencing of mRNA transcripts evidenced the mutation in homozygosis in the index patient (Figure 1E).

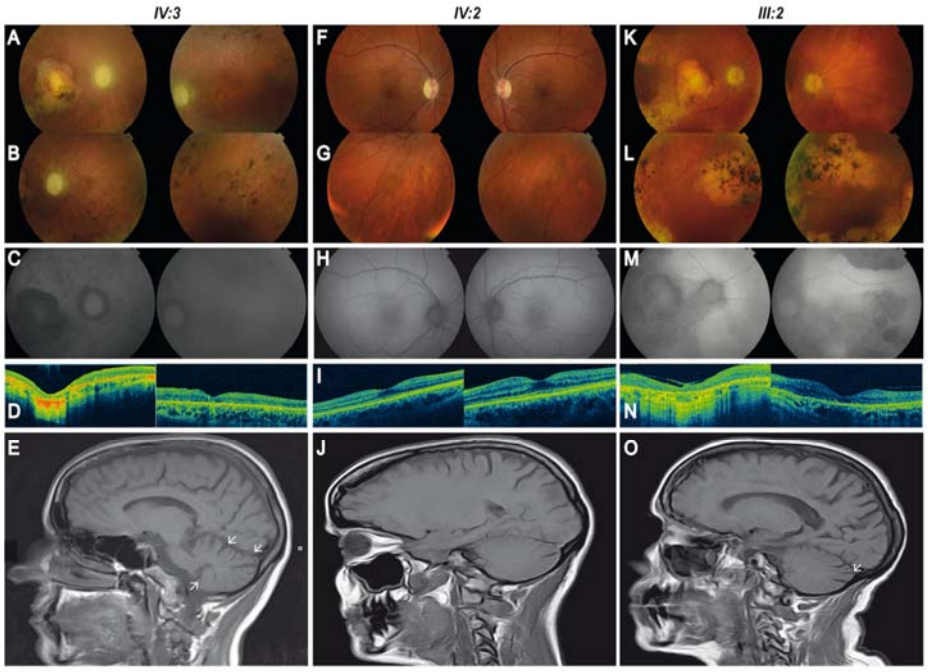

Figure 2 Retinal and head MRI imaging of patients carrying the p.Ser16Pro variant in *PRPS1*. A-E. Retinal imaging of both eyes (BE) of the proband IV:3 at age 35. **(A-B)** Fundus photographies show pale and atrophic papilla, narrowed vessels, diffuse hypopigmentation, bone spicules, and retinal pigment epithelium (RPE) atrophy in the mid-periphery of BE, and a well-defined atrophic macular lesion in the right eye (RE). **(C)** Autofluorescence reveals hypofluorescence areas corresponding with pigmented spicules and atrophic lesions. **(D)** Optical coherence tomography (OCT) confirms the generalized atrophy in all retinal layers in both perifoveal and foveal regions in RE. Well-conserved retinal architecture was found in the left eye (LE) and the atrophy is restricted to RPE and external layers. **(E)** Head MRI scan of the proband IV:3 demonstrates a moderate cerebellar atrophy (white arrows). **F-J.** Retinal imaging of BE of IV:2 at age 37. **(F-G)** Fundus photographies show a slight pallor in the papilla, slight arteriolar attenuation mainly around the optic nerve, a normal macula and an incipient RPE atrophy. **(H)** Normal autofluorescence. **(I)** OCTs display atrophy of photoreceptors layer in the perifoveal region and normal foveal thickness. **(J)** Head MRI scan of individual IV:2 does not evidence any sign of cerebellar atrophy. **K-O.** Retinal imaging of BE of III:2 at age 70. **(K-L)** Fundus photographies evidence pale and atrophic papilla, substantially narrowed vessels, sparse pigmentation, patches of chorioretinal atrophy in the mid-periphery of BE, and a well-defined atrophic macular lesion in RE and RPE alteration in LE. **(M)** Autofluorescence imaging shows alternating areas of hypo and hyperfluorescence. **(N)** OCT shows subfoveal atrophy lesion with loss of retinal architecture, an epiretinal membrane in RE and defects in external layer in LE. **(O)** Head MRI scan of individual III:2 exhibits a mild cerebellar atrophy (white arrow).

Non-random patterns of X-chromosome inactivation in the index patient

Non-random patterns of X-chromosome inactivation were assessed in the three affecteds (III:2, IV:2 and IV:3) and one unaffected individual (IV:1). Only in IV:3, there was a significantly skewed inactivation (82%) of the paternal allele, which explains the lack of expression of the wild type allele observed in the mRNA (Figure 1E). Individuals IV:1, IV:2 and III:2, had 50%, 61% and 64% X-chromosome inactivation, respectively, so they were considered non-skewed.

Discussion

Here we report a novel mutation in *PRPS1* leading to PRS-I deficiency in three-females from a family with a phenotype consisting of OA followed by RP in all cases, plus neurological features overlapping CMTX5 and Arts syndrome with variable presentation in the proband (IV:3) and her mother (III:2).

Despite being already described that carrier females of *PRPS1* mutations can exhibit PRS-I deficiency and a disease phenotype [3,7-9], to date, no female has been reported to display such a complex and severe phenotype

Table 1 Summary of findings in family RP-0482 and comparison with other PRS-I deficiency disorders (adapted from de Brouwer et al. [11])

	IV:3	IV:2	III:2	Arts syndrome	CMTX5	DFN2
Date of birth	1978	1976	1943			
Symptoms (age at diagnosis)						
Ophthalmological						
Retinitis Pigmentosa	+ (14y)	+ (23y)	+ (47y)	-	-	-
Night blindness	++ (4y)	+ (16y)	+	-	-	-
Visual field constriction	++	+	+	-	-	-
Visual acuity loss	++ (4y)	-	++	-	-	-
ERG alteration	++	+/–	++	-	-	-
Pigmentary changes at fundus	+	+/–	+	-	-	-
Macular atrophy	++	+	+	-	-	-
Optic atrophy	+ (5y)	+ (16y)	+ (47y)	+	+ (~10y)	+
Nystagmus	+ (Congenital)	-	-	-	-	-
Cataracts	+	-	+	-	-	-
Hyperopia	+	+	+/–	-	-	-
Hearing impairment	++ (21y)	-	+ (50y)	++	+	+
Neurological	++ (34y)	-	+ (55y)			
Mild developement delay	+/–	-	-	+	-	-
Hypotonia	+	-	-	+	+	-
Delayed motor development	-	-	-	+	-	-
Peripheral neuropathy	++	-	+	+	+	-
Pes cavus	+	-	-	NR	NR	NR
Loss of deep tendom reflexes	-	-	-	-	+	-
Cerebellar Atrophy	++	-	+			
Ataxia	+	-	+	+	+	-
Essential tremor	+	+	+			
Symptoms in carrier females	+++	+	++	Isolated and milder	Hearing loss	None
PRS activity erythrocytes (nmol/h/mg Hb) (Reference: 70–126)	Erythrocytes: 10	Erythrocytes: 41	Erythrocytes: 65	Erythrocytes: No activity Fibroblasts 13-fold decrease EBV-LCLs: normal	Fibroblasts: Decreased	Erythrocytes and fibroblasts: Decreased
Structural effect of mutation	Whole protein structure?			ATP site and allosteric sites I and II	ATP site and allosteric site I	Local structure

The number of "+" indicate the severity of the manifestation. NR = Not reported and EBV-LCLs = Epstein-Barr virus–transformed lymphoblastoid cell lines.

as observed in this family, specially in the proband. This patient showed a phenotype with features of Arts syndrome and CMTX5, with optic atrophy as the first symptom, followed by RP and neurological manifestations, such as hearing loss, intellectual disability and peripheral neuropathy (Table 1). The findings from this patient are consistent with the increasing evidence of intermediate phenotypes in PRS-I syndromes, recently described by several authors [6,7,12,37]. In particular, Synofzik and colleagues recently described a male with an intermediate phenotype between CMTX5 and Arts syndrome and a carrier female affected with DFN2 due to X-chromosome inactivation skewing [7]. The authors observed a correlation between the enzymatic residual activity, the degree of X chromosome inactivation skewing and the phenotype in the female and postulated that the location of the mutation and the residual enzymatic activity would be the main determinants of the phenotypic manifestations in males and females, respectively [7]. The three affected females from our family exhibited different levels of PRS deficiency in erythrocytes, ranging from a severe decrease in the activity in IV:3 to an

almost normal activity in III:2. However, non-random patterns of X-chromosome inactivation were exclusively observed for the index patient (IV:3), who was found to only express the mutant allele in lymphocytes. In this patient, there was an apparent correlation between the X-chromosome inactivation in leukocytes, the lack of expression of the wild type allele in lymphocytes, the degree of PRS deficiency in erythrocytes, and the severity of the phenotype. X chromosome inactivation in subjects III:2 and IV:2 was not skewed and the degree of PRS-I deficiency only correlated with the age of onset of ophthalmological symptoms but not with the presentation or severity of the phenotype. This lack of correlation is not surprising given that X-inactivation, whether random or biased, occurs in a tissue-specific manner and what is observed in a particular tissue or cell type, such as leukocytes may not be representative of the status of central and peripheral nervous systems [7,38], which are primarily affected in PRS-I deficiency syndromes. Given the variable expression of the disease in the family it is very possible that either X-inactivation is skewed in those target systems or that the expression of PRS-II is compensating PRS enzymatic function as previously suggested, and so phosphoribosyl pyrophosphate synthesis would not be critically affected [11].

A relationship between the level of PRS-I disruption and the different syndromic manifestations has been suggested [11], with the most severe syndromes caused by mutations predicted to impact allosteric and active sites and those responsible for milder phenotypes disrupting the structure locally [11]. Very recently, Al-Maawali and colleagues expanded the *PRPS1* phenotype to retinal dystrophy and diabetes insipidus in a family with two males affected with Leber's congenital amaurosis along with other manifestations, and no carrier female affected [37]. The mutation responsible for the phenotype was located in exon 5 of the gene (p.Arg196Trp), close to the ATP binding site of PRS-I. Residue Ser16 is located in the N-terminal domain of the protein, in a region not previously found mutated (Figure 1C) and far from the allosteric and active sites, both associated with Arts syndrome, CMTX5, and the recent retinal dystrophy phenotype, all features harbored by this family. That demonstrates once again the mutation-specific nature of PRS-I phenotypes [7,11]. Nevertheless, the existence of a putative defective splicing isoform, as predicted by *in silico* analysis cannot be ruled out, as it would be degraded by nonsense-mediated decay and thus, would not be detected by RT-PCR. In addition, other genes or epigenomic factors could be contributing to the complexity and severity of the phenotypes of PRS-I syndromes. Finally, it is worth noting that we cannot rule out either a dominant inheritance pattern for this particular mutation with a lethal effect in hemizygotes due to the lack of affected males in the family.

Conclusion

These results support previous findings evidencing the existence of intermediate phenotypes in PRS-I deficiency syndromes and demonstrate that female carriers of *PRPS1* mutations can be as severely affected as their male counterparts and therefore, these syndromes may need also to be considered in females even in the absence of affected males in the family.

Additional files

Additional file 1: Clinical data of family RP-0482 and primers used in the study.

Additional file 2: Figure S1. Multifocal ERG and Optical coherence tomography (OCT) findings suggested a sectorial affectation of perifoveal photoreceptors. A. Right eye multifocal ERG (mfERG) records in patient IV:2 demonstrate a reduced function of photoreceptors of perifoveal region with preserved function of fovea. The ring analysis (see schematic) goes from the centre to the periphery. Quantitative results of mfERG analyses are displayed in table format. B. OCT macular cube 512×128 scan show macular thickness in the right eye from patient IV:2. ILM: inner limiting membrane; RPE: retinal pigment epithelium. Top left: fundus image with scan cube overlay. Top right: macular thickness significance map. The central innermost 1-mm-diameter circle represents the central subfield; inner superior, inner nasal, inner inferior, and inner temporal areas bounded by the 3-mm-diameter circle form the inner macula; outer superior, outer nasal, outer inferior, and outer temporal areas bounded by the 6-mm-diameter circle form the outer macula. Retinal thickness values from ILM to RPE are compared to the normative data. Middle and bottom left: cross-sectional OCT scans. Middle right: 3days surface maps: the ILM-RPE, displaying the retinal thickness in three dimensions. Bottom right: central subfield thickness, overall average macular thickness, and overall macular volume compared to normative data are displayed in table format. Reduction in the retinal thickness in the perifoveal region with a normal foveal thickness is also evidenced.

Additional file 3: Table S1 and S2. Genetic variants obtained (single nucleotide variants –SNV-, Table S1, and insertion deletion variants –indels-, Table S2) after the first variant filtering analysis. **Table S3.** Variants yielded by the second filtering of the 141 variants based on the novelty, conservation across species according to the values from PhyloP and GERP++, predicted pathogenicity at least by two of the systems evaluated and expression in the retina. 1KGP: Minor allele frequency in the 1000 Genome Project, ESP: Minor allele frequency in the NHLBI GO Exome Sequencing Project. For PhyloP prediction, C: Conserved, NC: Not conserved. For pathogenicity prediction systems, B: benign, D: deleterious (LRT), or damaging (SIFT) or disease causing (MutationTaster), N: neutral (LRT) or polymorphism (MutationTaster), NA: missing data, PrD: probably damaging, PsD: possibly damaging, T: tolerated, U: Unknown.

Abbreviations

CMTX5: X-linked Charcot-Marie-Tooth, type 5; DFN2: X-linked non-syndromic sensorineural deafness; Indel: Insertion/deletion polymorphism; OA: Optic atrophy; PRPP: Phosphoribosyl pyrophosphate; PRS: Phosphoribosyl pyrophosphate synthetase; RP: Retitinis pigmentosa; SNV: Nonsynonymous single nucleotide variant; WES: Whole exome sequencing.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BA contributed to the design of the study, performed experiments and data analysis, the interpretation of results, and wrote the manuscript. SH contributed to the design of the study, performed the whole exome sequencing and helped with the data analysis. MC and PFS contributed to the design of the study, performed experiments and data analysis, interpretation of the results and wrote the manuscript. FBK, MILM, BGS, JdV

performed the clinical evaluation of the patients and the interpretation of the phenotypic analysis. YG and LT contributed to the design of the study, performed data analysis and interpretation of the results. XL, LG, and XX performed the whole exome sequencing and helped with the data analysis. RJT and JGP performed experiments, data analysis and interpretation of results. HH contributed to the design of the study, interpretation of the results and writing of the manuscript. BK contributed to the design of the study and helped in the data analysis. CA contributed to the design of the study, the clinical evaluation of the patients; the interpretation of the results and the writing of the manuscript. All authors contributed to the writing and critical review of this manuscript. All authors read and approved the final manuscript.

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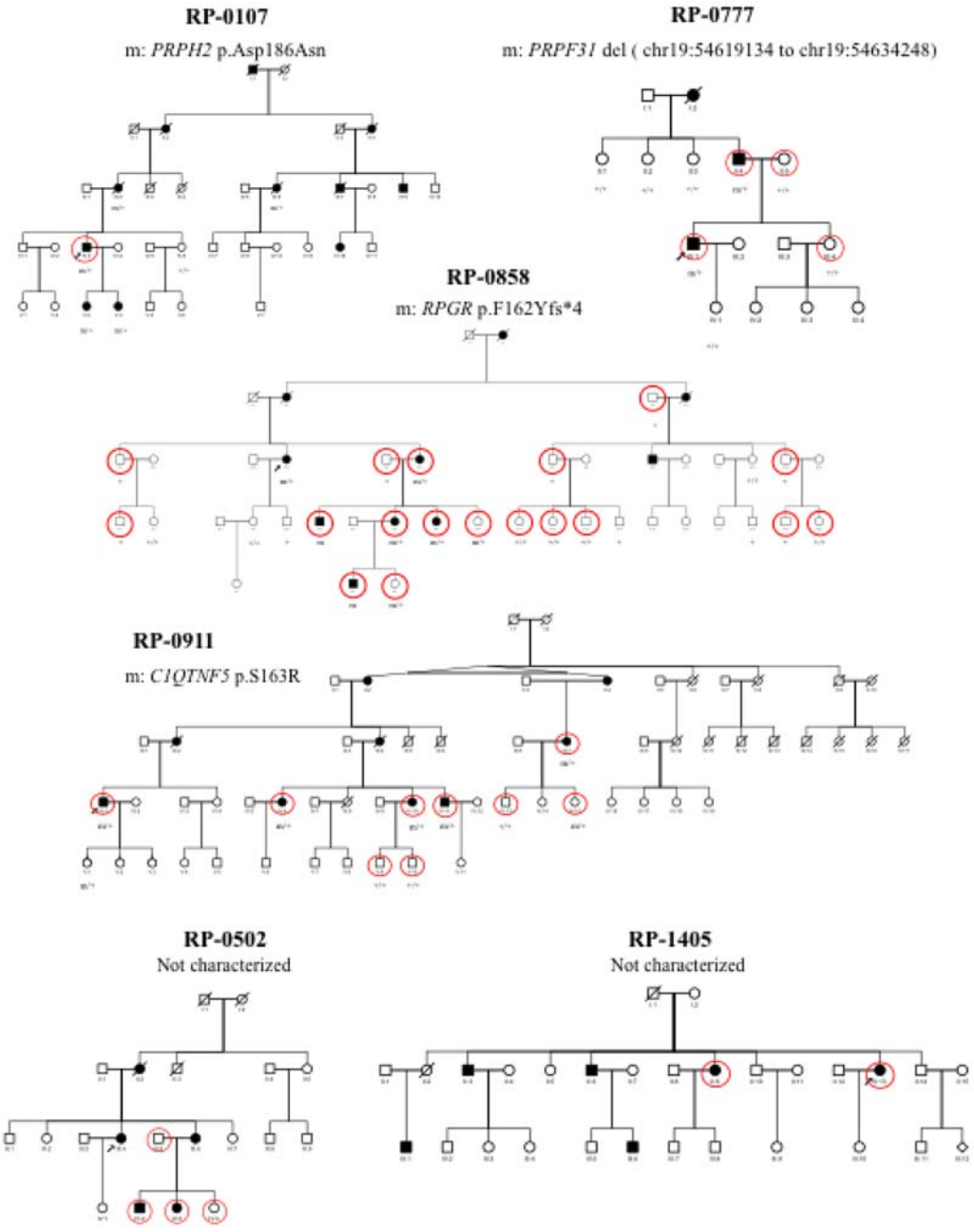
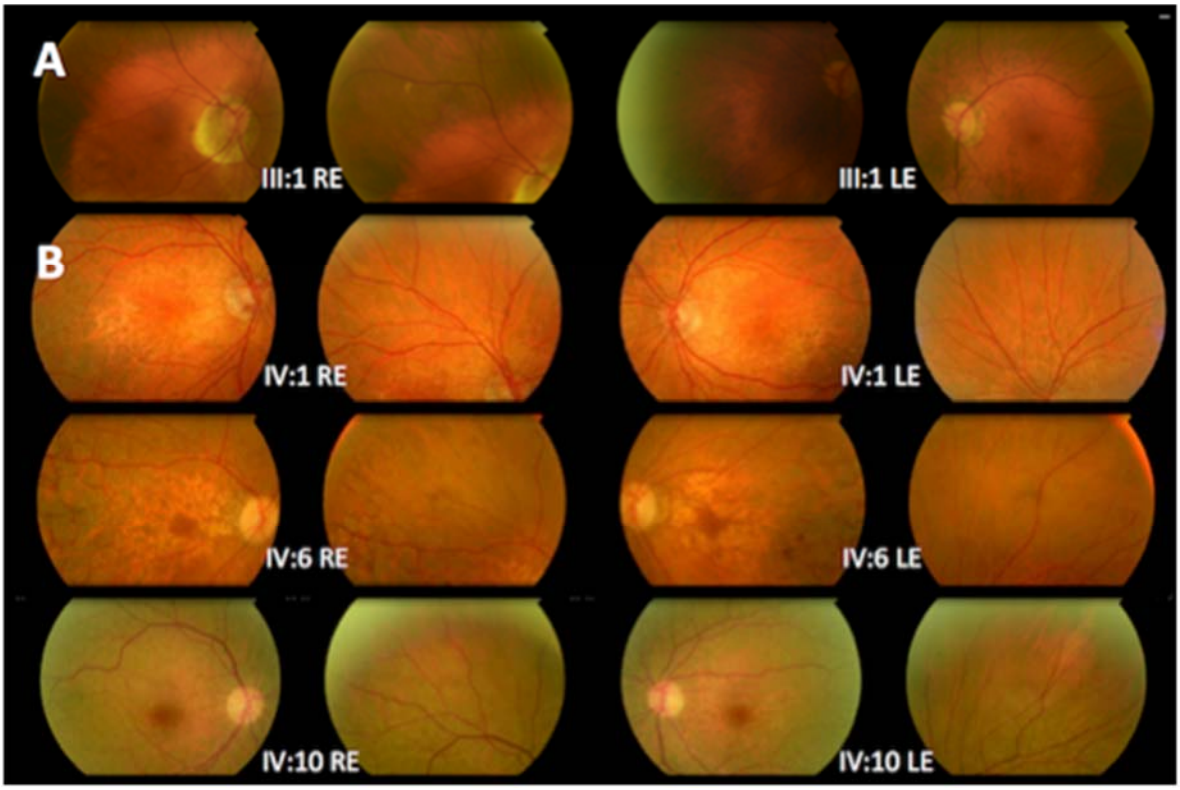
Supporting information

S1 Fig. Pedigrees of the six families studied. + and +/+: wild type genotypes. m: mutation detected in hemizygosis; m/+: mutation detected in heterozygosis. Filled and unfilled symbols represent affected and unaffected individuals respectively. Squares indicate males and circles females. Arrows indicate the index cases. Red circles represent individuals subjected to whole exome sequencing.

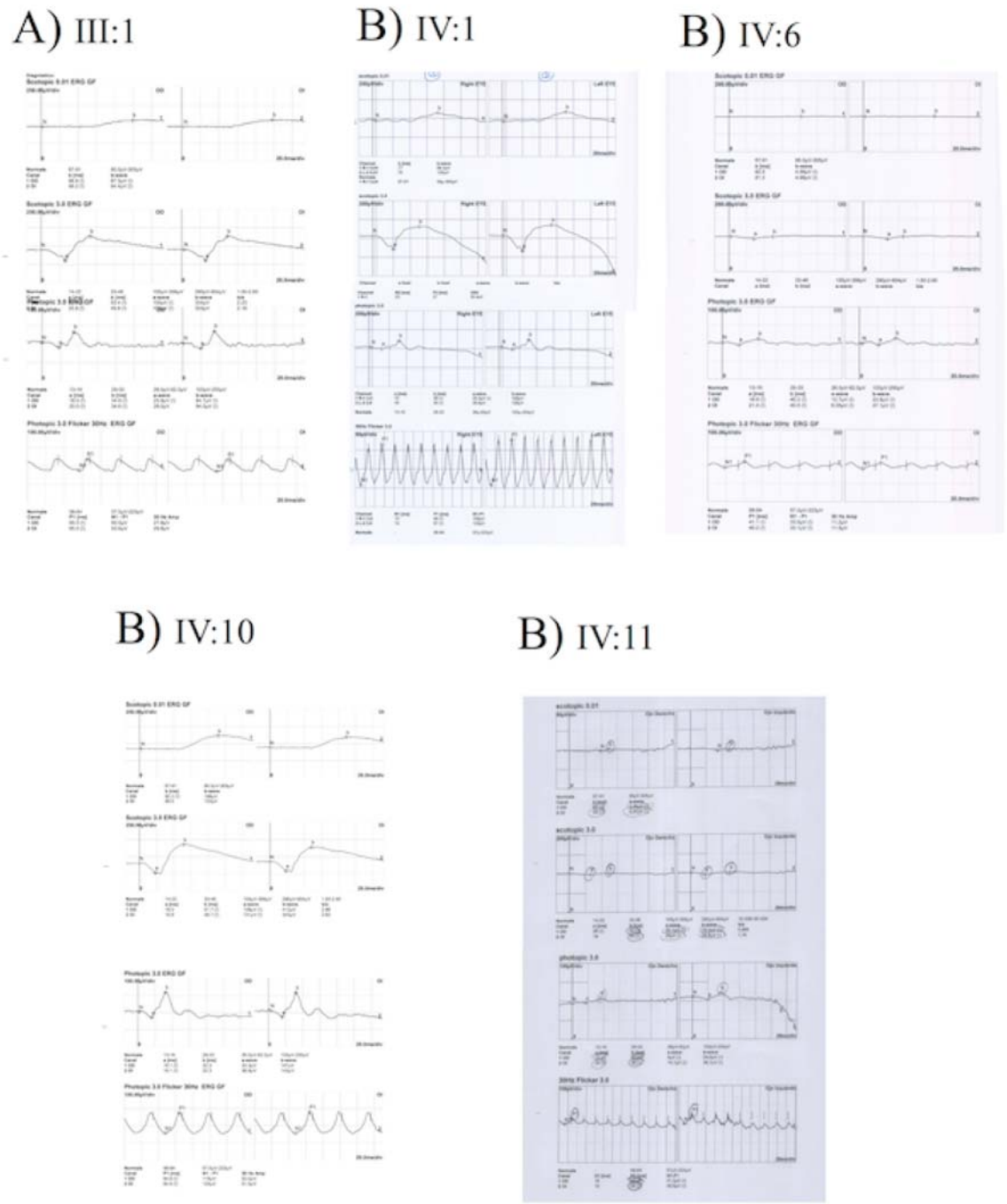
Artículo: Expanding the phenotype of PRPS1 syndromes in females: neuropathy, hearing loss and retinopathy.



S2 Fig. Eye fundus images from members of families RP-0777 and RP-0911. Each patient presents two fundus images per eye. A) Family RP-0777 and B) Family RP-0911. RE = Right eye. LE = Left eye.



S3 Fig. Electroretinogram recordings from members of families RP-0777 and RP-0911.



S1 Table. Genetic screening performed to the six families prior to whole exome sequencing. SSCP: Single Strand Conformation Polymorphism; DGGE: CG-clamped Denaturing Gradient Gel Electrophoresis. The parentheses indicate the exons targeted by these techniques; otherwise the entire gene was screened. For ADRP Chip , version 1 includes 355 SNPs in *CA4, CRX, FSCN2, IMPDH1, NR2E3, NRL, PRPF3, PRPF31, PRPF8, PRPH2, RHO, ROM1, RP1, RP9, TOPORS*; and version 2 includes 414 SNPs in *CA4, CRX, FSCN2, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF31, PRPF8, PRPH2, RHO, ROM1, RP1, RP9, TOPORS*; Sanger sequencing was used to screen mutations in exons 16 and 25 for *SNRNP200*, exon 2 for *NR2E3* and exon 13 for *GUCY2D*. For IMPDH1 all exons were sequenced. RD_NGS_Panel refers to the custom Next Generation Sequencing panel from S2 Table.

	SSCP/DGGE	**ADRP Chip® version**	**Genes subjected to Sanger sequencing**	**RD_NGS_Panel**
RP-0107	*CA4 (3, 4, 6), CRX, ELOVL4, FSCN2, NRL PRPF3, PRPF8 (42), PRPF31 (8), PRPH2, RHO, ROM-1*	Version 2	*IMPDH1, SNRNP200*	No
RP-0502	*CRX, ELOVL4, FSCN2, IMPDH1, PRPH2, RHO, ROM-1, NRL, PRPF8 (42), PRPF31 (8)*	Version 1	*IMPDH1, NR2E3, PRPH2, SNRNP200*	Yes
RP-0777	*CA4 (3, 4, 6) CRX,PRPH2, RHO, PRPF8 (42), PRPF31 (8)*	Version 2	*IMPDH1, NR2E3, PRPH2, SNRNP200*	No
RP-0858	*CA4 (3, 4, 6), CRX,PRPH2,RHO, PRPF8 (42), PRPF31 (8)*	Version 2	*IMPDH1, NR2E3, PRPH2, SNRNP200*	No
RP-0911	*CA4 (3, 4, 6), CRX,PRPH2, RHO, PRPF8 (42), PRPF31 (8)*	Version 1	*GUCY2D, IMPDH1, NR2E3, SNRNP200*	No
RP-1405	NA	Version 2	*GUCY2D, IMPDH1, NR2E3, SNRNP200*	Yes

!

S2 Table. Genes associated with RP and LCA included in the customized RD_NGS_Panel. Macular dystrophy (MD); retinitis pigmentosa (RP); Leber's congenital amaurosis (LCA); congenital stationary night blindness (CSNB); choroideremia (CHM), cone-rod dystrophy (CORD); autosomal recessive (ar); autosomal dominant (ad); X-linked (xl); McKusick-Kaufman syndrome (MKKS); Senior Loken Syndrome (SLS); vitreoretinopathy proliferative (VRP); enhanced S-cone syndrome (ESC).

Gene	cDNA sequence	Number of targets	% Coverage of design	Disease	Inheritance	Other described phenotype
ABCA4	NM_000350.2	50	100%	MD	ar	arRP/arCORD
ABHD12	NM_001042472.2	14	100%	RP	ar	RP+ataxia+hearing loss
ADAMTS18	NM_199355.2	23	99.6%	RP	ar	
AIPL1	NM_014336.3	6	95.6%	LCA	ar	adCORD
BBS1	NM_024649.4	17	100%	ciliopatia	ar	Bardet-Biedl / arRP
BEST1	NM_004183.3	11	100%	MD	ad / ar	arRP / adRP / ad vitreoretinochoroidopathy/ ar bestrophinopathy
C2orf71	NM_001029883.2	2	100%	RP	ar	
C8ORF37	NM_177965.3	6	100%	RP	ar	CORD
CA4	NM_000717.3	8	97.0%	RP	ad	
CABP4	NM_145200.3	6	98.3%	CSNB	ar	arCORD / LCA
CEP290	NM_025114.3	55	99.4%	LCA	ar	SLS/ Joubert / MKKS
CERKL	NM_001030311.2	14	98.0%	RP	ar	arCORD with inner retinopathy
CHM	NM_000390.2	16	99.0%	CHM	xl	
CLRN1	NM_174878.2	5	100%	Usher	ar	arRP
CNGA1	NM_001142564.1	12	99.9%	RP	ar	
CNGB1	NM_001297.4	34	99.9%	RP	ar	
CRB1	NM_201253.2	12	100%	LCA	ar	arRP
CRX	NM_000554.4	4	95.5%	LCA	ar / ad	adRP / adCORD
CYP4V2	NM_207352.3	11	97.6%	RP	ar	Bietti crystalline corneoretinal dystrophy
DHDDS	NM_024887.3	9	100%	RP	ar	
EYS	NM_001142800.1	45	99.2%	RP	ar	
FAM161A	NM_032180.2	7	100%	RP	ar	
FSCN2	NM_001077182.2	5	100%	RP	ad	
GUCA1B	NM_000180.3	4	96.2%	RP	ad	
GUCY2D	NM_000180.3	20	98.5%	LCA	ar	adRP / adCORD
IDH3B	NM_006899.3	12	100%	RP	ar	
IMPDH1	NM_000883.3	17	100%	RP	ad	
IMPG1	NM_001563.2	17	100%	RP	ar	
IMPG2	NM_016247.3	19	99.7%	RP	ar	
IQCB1	NM_001023570.2	15	99.8%	LCA	ar	SLS
KCNJ13	NM_002242.4	3	100%	LCA	ar	adVRP
KLHL7	NM_001031710.2	14	100%	RP	ad	
LCA5	NM_181714.3	9	99.9%	LCA	ar	
LRAT	NM_004744.3	3	97.8%	RP	ar	LCA

!

Gene	cDNA sequence	Number of targets	% Coverage of design	Disease	Inheritance	Other described phenotype
MAK	NM_001242957.1	13	98.2%	RP	ar	
MERTK	NM_006343.2	19	100%	RP	ar	LCA / arCORD
MPDZ	NM_003829.4	45	100%	RP	ar	
NMNAT1	NM_022787.3	5	93.4%	LCA	ar	
NR2E3	NM_014249.2	8	100%	RP	ar / ad	arESC; Goldmann-Favre syndrome; combined adt and ar retinopathy
NRL	NM_006177.3	3	100%	RP	ar / ad	
OFD1	NM_003611.2	24	100%	XLRP	xl	Joubert
PDE6A	NM_000440.2	22	98.4%	RP	ar	
PDE6B	NM_000283.3	23	99.5%	RP	ar	ad CNSB
PDE6G	NM_002602.3	4	100%	RP	ar	
PRCD	NM_001077620.2	6	100%	CORD	ar	arRP
PROM1	NM_006017.2	29	100%	MD	ad / ar	arRP/ adCORD
PRPF3	NM_004698.2	16	100%	RP	ad	
PRPF31	NM_015629.3	15	97.4%	RP	ad	
PRPF6	NM_012469.3	21	100%	RP	ad	
PRPF8	NM_006445.3	43	99.8%	RP	ad	
PRPH2	NM_000322.4	3	100%	RP	ad	adMD / adCORD / digenic retinitis pigmentosa with ROM1
RBP3	NM_002900.2	4	100%	RP	ar	
RD3	NM_183059.2	3	100%	LCA	ar	
RDH12	NM_152443.2	9	100%	LCA	ar / ad	arRP/adRP
RGR	NM_001012720.1	7	91.0%	RP	ar	ad choroidal sclerosis
RHO	NM_000539.3	5	100%	RP	ad / ar	ad CNSB
RLBP1	NM_000326.4	9	100%	RP	ar	arCORD / ar Bothnia dystrophy / ar retinitis punctata albescens
ROM1	NM_000327.3	3	100%	RP	ad	digenic retinitis pigmentosa with PRPH2
RP1	NM_006269.1	4	100%	RP	ad / ar	
RP2	NM_006915.2	5	98.4%	RP	xl	
RP9	NM_203288.1	6	84.6%	RP	ad	
RPE65	NM_000329.2	14	98.0%	LCA	ar	arRP
RPGR	NM_001034853.1	15	84.8%	RP	xl	xlCORD / xlMD
RPGRIP1	NM_020366.3	24	100%	LCA	ar	arCORD
SAG	NM_000541.4	16	99.6%	RP	ar	CNSB / ar Oguchi disease
SEMA4A	NM_022367.3	16	100%	RP	ad	adCORD
SNRNP200	NM_014014.4	45	99.6%	RP	ad	
SPATA7	NM_018418.4	12	98.9%	LCA	ar	arRP
TOPORS	NM_005802.4	3	100%	RP	ad	
TTC8	NM_144596.2	15	100%	RP	ar	Bardet-Biedl syndrome

S3 Table. Primers and conditions used for PCR and sequencing of *ORF15* * An internal primer for sequencing of amplicon 11 was also used.

Amplicon	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size (bp)	PCR Annealing Temperature (ºC)
1	GTAGATAAGTTGTCCTTGTC	TTCGATAGTCGTAGCTGGC	226	54
2	AATGAAAGAAGGGAAAGCATG	TGCTTCTGATTCCTTCTGAC	258	55
3	GGTGTTGATCAACTTGATG	CACATCTTGCTTGCAACTTTC	264	54
4	GAGGTATGAATACTGAGAGTG	ATTTCCTGCCATACCGTATG	249	55
5	ATGGATTCCAGCAGCCTGAG	CCTTTTGAATCCTCTGCTCC	203	58
6	GATCGCTTGTCAGAGATCCC	ACTACCTTCCTCACAGTTC	249	56
7	AGAAGCAGAGGATGGGCCTG	CTTTCCTTCTGATGGCCCTG	234	62
8	GAATGGAAGAAGAGGGATGG	TTTTCACGTTCTCCCTCCAC	219	56
9	GGAGGAGGAGCATGGAGAAG	CCTCTTCCCCCTCTCCTTGG	203	58
10	GTGGAGGGAGAACGTGAAAAG	CCCTCCCCTTCCTCCTCTTC	236	67
11	AGGAGAGGAAGAAGGAGACC	TTCTTCGCCTGTCTCCTGATA	868	63
11_int *	----	TTCCCCCTCCCCTTCTCCA		
12	GATCTGTGAAATATGGCAAAC	GACTGGCCATAATCGGGTCAC	215	54
13	GGAGGAAGAAGAGGGGAAGTATCA	CCCTGTGTGTTAGTAACTGAC	154	58

Artículo: *Application of Whole Exome Sequencing in Six Families with an Initial Diagnosis of Autosomal Dominant Retinitis Pigmentosa: Lessons Learned*

En este estudio, hemos tratado de identificar nuevos genes y mutaciones responsables de distrofia de retina mediante secuenciación exómica completa. Para ello se seleccionaron 6 familias con un diagnóstico clínico de retinosis pigmentaria autosómica dominante que previamente habían sido cribadas para los genes y mutaciones conocidos asociados a adRP mediante técnicas convencionales de genética molecular.

Las variantes tipo SNVs e indelsdetectadas mediante *WES* fueron priorizadas y analizadas teniendo en cuenta cambios de tipo *missense*, *LOF* y en sitios canónicosde *splicing*, y, de ellas, se seleccionaron las variantes nuevas y aquellas con una MAF<0.1%. Además fueron priorizadas las variantes detectadas en genes previamente asociados a distrofias de retina y en nuevos genes con expresión en la retina. Finalmente, se evaluó la segregación de cada variante candidata con la enfermedad en los familiares. Este análisis de SNVs e indels nos permitió identificar: i) una mutación previamente descrita en *PRPH2* asociada a una forma dominante de distrofia de conos-bastones; ii) una deleción nueva en el gen *RPGR* asociada a un modelo de herencia ligado al cromosoma X y iii) un cambio nuevo en *C1QTNF5* asociado a una degeneración retiniana de inicio tardío (*Late-Onset Retinal Degeneration,* LORD); lo que permitió, por tanto, la reclasificación clínica y/o genéticade estas tres familias.

Por último, en los casos negativos, se hizo un estudio de variación en el número de copias (*Copy Number Variants, CNVs*) utilizando los datos de NGS. Se identificó una gran deleción en heterocigosis que abarcaba todo el gen *PRPF31* mediante el análisis de *CNVs*, que fue validada mediante *qPCR*, siendo este uno de los primeros trabajos publicados en los que se detectaron deleciones utilizando datos de secuenciación exómica completa.

Por lo tanto, en este trabajo se pudo caracterizar genéticamente a 4 de 6 familias estudiadas utilizando la tecnología de *WES*. Este paso es fundamental para establecer un diagnóstico correcto, estimación del riesgo de recurrencia, pronóstico y adecuado consejo genético para los pacientes y sus familias. Además, tres de las familias fueron reclasificadas por lo que este resultado pone de manifiesto un problema relativamente común en este tipo de patologías que presentan una clínica solapante y en la que, a veces nos encontramos con

escasa información clínica de la familia como para hacer una clasificación inicial precisa de la patología.

Finalmente, como todos los cambios fueron detectados en genes conocidos asociados a distrofias de retina, concluimos que la secuenciación masiva basada en un panel de genes parece la estrategia más adecuada en la actualidad, por su menor coste y manejo más sencillo, para abordar el estudio de las familias con adRP. En una segunda fase, el uso de la secuenciación del exoma completo en casos negativos representa un abordaje óptimo para identificar nuevos genes asociados a distrofias de retina y ampliar el diagnóstico molecular de pacientes con distrofia de retina.

Aportación de la autora

En este trabajo la autora ha estado implicada en la selección, recopilación y/o recogida de muestras y datos clínicos y genéticos de las 6 familias incluidas en este estudio. Fue la encargada de validar y segregar mediante secuenciación Sanger las variantes encontradas en los 66 individuos de las 6 familias de las que disponíamos ADN, así como el cribado de la región *ORF15* del gen *RPGR* en una de las familias. También colaboró en el filtrado, priorización, análisis e interpretación de los resultados obtenidos mediante *WES* y estuvo implicada en la revisión de la información oftalmológica de 17 pacientes utilizada en las correlaciones fenotipo-genotipo presentadas en este trabajo. Finalmente la doctoranda contribuyó en la redacción del manuscrito.

PLOS | ONE

RESEARCH ARTICLE

Application of Whole Exome Sequencing in Six Families with an Initial Diagnosis of Autosomal Dominant Retinitis Pigmentosa: Lessons Learned

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Abstract

This study aimed to identify the genetics underlying dominant forms of inherited retinal dystrophies using whole exome sequencing (WES) in six families extensively screened for known mutations or genes. Thirty-eight individuals were subjected to WES. Causative variants were searched among single nucleotide variants (SNVs) and insertion/deletion variants (*indels*) and whenever no potential candidate emerged, copy number variant (CNV) analysis was performed. Variants or regions harboring a candidate variant were prioritized and segregation of the variant with the disease was further assessed using Sanger sequencing in case of SNVs and *indels*, and quantitative PCR (qPCR) for CNVs. SNV and *indel* analysis led to the identification of a previously reported mutation in *PRPH2*. Two additional mutations linked to different forms of retinal dystrophies were identified in two families: a known frameshift deletion in *RPGR*, a gene responsible for X-linked retinitis pigmentosa and p.Ser163Arg in *C1QTNF5* associated with Late-Onset Retinal Degeneration. A novel heterozygous deletion spanning the entire region of *PRPF31*was also identified in the affected members of a fourth family, which was confirmed with qPCR. This study allowed the identification of the genetic cause of the retinal dystrophy and the establishment of a correct diagnosis in four families, including a large heterozygous deletion in *PRPF31*, typically considered one of the pitfalls of this method. Since all findings in this study are restricted to known genes, we propose that targeted sequencing using gene-panel is an optimal first approach for the genetic screening and that once known genetic causes are ruled out, WES might be used to uncover new genes involved in inherited retinal dystrophies.

OPEN ACCESS

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Introduction

Despite the advances over the last decades on the genetics of inherited retinal dystrophies, molecular diagnosis of this heterogeneous group of diseases is still challenging. The inherited retinal dystrophies include a wide spectrum of diseases caused by more than 190 genes identified so far (RetNet; https://sph.uth.edu/retnet/), and represent the most frequent cause of genetic blindness in the Western world. With an overall prevalence of up to 1 in 4000 individuals worldwide [1], retinitis pigmentosa (RP) is the most common form of inherited retinal dystrophy and accounts for almost half of the patients [2].

RP is a set of inherited progressive and degenerative retinal diseases that lead to loss of vision (reviewed in [2] and [3]). All types of Mendelian inheritance patterns have been described for RP, with autosomal dominant RP (adRP) accounting for 15–25%, autosomal recessive RP (arRP) for 35–50%, and X-linked RP (xlRP) for up to 15% of the families [4]. One particular characteristic of RP is its extreme genetic and allelic heterogeneity, what makes the diagnosis of patients a complex task. As of the last update of the Retinal Information Network (RetNet; https://sph.uth.edu/retnet/), more than 2,800 mutations in 81 genes had been identified to cause RP: 26 in adRP, 52 in arRP and three causing xlRP. To make it even more complex, large phenotypic heterogeneity is also observed, with mutations in the same genes causing different diseases and the same mutation displaying extensive variation in clinical expression, if not clinically distinct entities, among individuals [3]. Symptoms and phenotypes are variable between families and also in different members of the same family, and several genes display incomplete penetrance [2].

Until the advent of Next Generation Sequencing (NGS), molecular diagnosis of RP was mainly based on a combination of arrayed primer extension (APEX) technology of previously known mutations, and Sanger sequencing, which results unaffordable for the screening of all potentially causative genes. Due to its high-throughput nature, NGS is revolutionizing the way disease-causing mutations of Mendelian disorders are identified [5], [6] being able to simultaneously scan multiple genes in a cost-efficiently manner and has proven very productive in RP, with high mutation detection and diagnostic rates [7–11], as well as the discovery of new genes [12–14]. Specifically, targeted capture of known and candidate genes is emerging as the most optimal diagnostic tool for RP [10, 11, 15, 16] with a mutation detection rate of 20–70%, depending on the inheritance pattern and selection criteria [3], [7, 8]. Notably, approximately 50% of the cases of adRP are estimated to harbor mutations in novel genes [3] that might not be captured by gene-panels, and in these cases whole exome sequencing (WES), which targets the complete coding part of the genome, could help identify the missing causative genes. In this study, we used WES in six families with a suspected diagnosis of adRP as a part of a larger effort on the identification of the genetic causes of Mendelian disorders. The application of WES led to the characterization of four of the six families, allowing the reappraisal of the diagnosis in three, and the identification of a novel large deletion in *PRPF31* responsible for the phenotype in a fourth family.

Materials and Methods

Subjects

A total of 66 individuals from six large unrelated Spanish families from the Fundacion Jimenez Diaz University Hospital, with an initial diagnosis of non-syndromic adRP were included in the current study (S1 Fig) The criteria for the assignment of autosomal dominant inheritance were based on that previously described by Ayuso *et al.* [17].

Thirty-eight members out of the 66 were selected for WES (S1 Fig). Written informed consent was obtained from all individuals involved in the study, and the research was performed in accordance with the tenets of the Declaration of Helsinki and further reviews. Protocols were approved by the Bioethics Committee of the IIS-Fundacion Jimenez Diaz.

Genomic DNA was extracted from peripheral blood lymphocytes and/or saliva (Oragene containers, DNA Genotek) using standard methods. Index cases enrolled were previously screened for known causes of adRP using a combined strategy of molecular tools: Single Strand Conformation Polymorphism (SSCP), CG-clamped Denaturing Gradient Gel Electrophoresis (DGGE), genotyping ADRP Chip (Asper Biotech, Tartu, Estonia), Sanger sequencing of prevalent adRP genes [18], [19], [20] and a NGS-based approach with a custom panel for 73 genes related to retinal dystrophies [10, 20]. (See S1 and S2 Tables).

Clinical evaluation

The clinical ophthalmic evaluation included the assessment of visual acuity (VA), intraocular pressure, ocular motility, pupillary reaction, biomicroscopic slit-lamp examination, and dilated fundus examination in all members of the six families. Visual function was performed by static perimetry, D15 panel testing, and Ganzfeld electroretinography according to the International Society for Clinical Electrophysiology of Vision (ISCEV) Standards [21] with a UTAS 2000 system (LKC Technologies, Gaithersburg, USA) and jet electrodes.

RP diagnosis was made in patients with night blindness, progressive visual field constriction, poor VA in advanced stages, and confirmed by altered or abolished electroretinogram (ERG) responses [22].

Whole exome sequencing

DNA samples were subjected to library construction using Agilent Sure Select Human All Exon kit version 2 covering 46MB of coding region (Agilent Technologies, Santa Clara, CA, USA), and sequenced on HiSeq 2000 instruments (Illumina, San Diego, CA, USA). Default parameters predefined in the Illumina sequencing workflow were applied to call bases from raw images, which produced raw sequencing reads that were mapped against the human reference genome (UCSC hg19), using the Burrows–Wheeler alignment tool [23]. Genome Analysis Tool Kit version 1.4 [24] was integrated with own scripts to design a variant calling pipeline for genomic variant detection, including single nucleotide variants (SNVs) and small insertions/deletions (*indels*). ANNOVAR [25] was used for variant functional annotation.

Variant prioritization

Causative variants were first searched among all SNVs and *indels* and whenever no potential candidate emerged, copy number variant (CNV) analysis was performed using WES data. Variants or regions harboring a candidate variant in genes previously associated with retinal dystrophies or with expression in the retina were prioritized and segregation of the variant with the disease was further assessed. Databases used for such prioritization were the Retinal Information Network Database (RetNet; https://sph.uth.edu/retnet/) and the Human Gene Mutation Database (HGMD; www.hgmd.org/).

SNVs and *indels* in coding regions and potentially functional (nonsynonymous, splice acceptor and donor site SNVs, or frameshift *indels*) were considered for the analysis. From those, only novel variants or those with a MAF<1% in a cohort of more than 8,000 control individuals (1,000 Genomes Project, April 2012 release; 6,503 exomes from NHLBI GO Exome

Sequencing Project-ESP6500SI; http://evs.gs.washington.edu/EVS/), and 669 in-house whole-exomes) were kept for the subsequent analyses.

CNV analysis was performed using the standard Exome Hidden Markov Model (XHMM) [26]. Briefly, target regions with extreme GC content (<10% or >90%), and low complexity regions were filtered out. Then, read depths of all targets and samples were calculated with GATK [24] and normalized using principal component analysis (PCA) to remove inherent biases in sample preparation and sequencing. Samples with extreme variability in normalized read depth were removed. Finally, per-sample CNV detection with a Hidden Markov Model was performed and quality metrics assigned to all samples for detected CNVs.

Genetic characterization of *ORF15* and molecular validation of the candidate variants

Sanger sequencing. Sanger sequencing was used to validate the candidate SNVs and *indels* selected, their segregation in the families and also to sequence the 3' end of a highly repetitive region of exon open reading frame 15 (*ORF15*) of RPGR in family RP-0502. All primers were designed using Primer3 (*frodo.wi.mit.edu/*). PCR products were enzymatically purified with ExoSAP-it (USB, Affymetrix), sequenced on both strands using Big Dye Terminator Cycle Sequencing Kit v3.1 Kit (Applied Biosystems) and resolved on an automated sequencer (ABI 3130xl Genetic Analyzer, Applied Biosystems).

For the mutation screening in *ORF15*, 13 primer sets were used for the amplification of exon 14 (*ORF14*) and exon 15 (*ORF15*) of RGPR (RefSeq NM_001034853) (S3 Table) [27]. PCR amplifications were done in 50-μL reactions using FastStart polymerase (Roche) according to the recommended protocols. PCR conditions were: 95°C for 5 minutes, followed by 35 cycles of pre-incubation at 95°C for 1 minute, annealing for 1 minute at the indicated temperature in S3 Table and extension at 72°C for 1 minute. After amplification, PCR products were enzymatically purified with ExoSAP-it (USB, Affymetrix) and sequenced on reverse strand using Big Dye Terminator Cycle Sequencing Kit v1.1 Kit (Applied Biosystems) in presence of 10% of betaine (Sigma). PCR products were purified on a 96-well multiscreen filter plate (Montage SEQ96 Sequencing Reaction Cleanup Kit, Millipore, Bedford, MA) and resolved on an automated sequencer (ABI 3130xl Genetic Analyzer, Applied Biosystems).

CNV validation. Validation of the large deletion in the gene *PRPF31* was performed in the two affected and six unaffected members of family RP-0777 using quantitative PCR (qPCR) with two different methods: TaqMan assays using the predesigned probes Hs01877341_cn (chr19:54,618,875) and Hs01993463_cn (chr19:54,619,056) (Applied Biosystems TaqMan Copy Number Assays, Life Technologies, Inc.) and the Universal Probe Library (UPL; Roche, Indianapolis, IN) with slight modifications of what was previously described in [28] and [29]. Briefly, primer and UPL probe combinations were designed against *PRPF31* genomic DNA sequence using the Probe Finder v2.49 software (Roche, Indianapolis, IN). Five assays spanning the length of the gene were selected for validation (genomic coordinates of each targeted amplicon listed in Table 1).

Quantitative PCR was performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Data were evaluated using the Sequence Detection Software v2.4 (Applied Biosystems, Foster City, CA) and further analyzed by the ΔΔCT method. The geometric mean of the CT values for the three control sequences (*GAPDH*, *RPPH1*, and *SNCA*) was calculated and used as the reference value for ΔCT calculations. Hemizygous deletions were determined when the relative copy number value for a specific sample normalized to the reference sample was less than 0.75.

Table 1. UPL Probes used for validation of *PRPF31* heterozygous deletion. The amplicon position is that reported by UCSC genome browser (hg19) *in silico* PCR tool. All primers are listed 5' to 3'.

Gene Symbol-Probe Name	Gene	Amplicon Position	UPL Probe #	Left Primer[2]	Right Primer[2]
PRPF31–345	PRP31 pre-mRNA processing factor 31 homolog	chr19:54,619,134–54,619,198 (exon 1)	55	ggtgagcgactaacgctagaa	cgtggtctccatcacactca
PRPF31–3068	PRP31 pre-mRNA processing factor 31 homolog	chr19:54,621,857–54,621,932 (intron 3)	14	ctagcaggggctctagaca	gtcagaatccagcactcttcaa
PRPF31–8679	PRP31 pre-mRNA processing factor 31 homolog	chr19:54,627,468–54,627,530 (intron 7)	7	gggaaaaacactcacccaca	gtggtcatctctgggtttcc
PRPF31–10932	PRP31 pre-mRNA processing factor 31 homolog	chr19:54,629,721–54,629,783 (intron 8)	17	ctgccctcatcccctctt	cccttgggctctagaggtgt
PRPF31–15386	PRP31 pre-mRNA processing factor 31 homolog	chr19:54,634,175–54,634,248 (intron 13)	25	cagtggctgtgcctttcc	gcttcctgtgcgttcttttc
RPPH1	RPPH1 ribonuclease P RNA component H1	chr14:20,811,245–20,811,337	30	ccggagcttggaacagact	gtagtctgaattgggttatgaggtc
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	chr12:6,645,563–6,645,625	10	gctgcattcgccctctta	gaggctcctccagaatatgtga
SNCA	Synuclein, Alpha	chr4:90,743,466–90,743,537	68	gctgagaagaccaaagagcaa	ctgggctactgctgtcacac

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Results

SNV and *indel* analysis with further confirmation of family segregation by Sanger sequencing allowed the genetic characterization of three families

Seventeen affected and 21 unaffected members from the six families were subjected to WES. A total of 107.7 GB of data on target genomic regions were generated for the 38 samples, with a mean coverage of target region of 63.67 fold (minimum coverage was 34.85 fold). An average of 50,562 SNVs and 8,476 *indels* were called for the 38 exomes, however, for further variant filtering only variants located in the coding regions and splicing boundaries were considered, thus reducing the number to an average of 18,278 SNVs and 708 *indels*.

Variant filtering was initially performed in the index cases from all six families and then segregation with the disease was assessed in the remaining family members analyzed by WES. SNV and *indel* analysis led to the identification of three previously reported mutations associated with retinal dystrophies in families RP-0107, RP-0858, and RP-0911. A description of the phenotypic features of the affected members of these families and figures with electroretinogram and eye fundus are shown in Table 2 and S2 and S3 Figs.

Family RP-0107 harbored a missense change in *PRPH2* (NM_000322.4:c556G>A; p.Asp186Asn) [30] and the complete segregation in all five affected and one unaffected members from RP-0107 was further confirmed by Sanger sequencing. RP-0858 carried a known *frameshift* deletion in *RPGR* (NM_000328.2: c.485_486delTT; p.Phe162Tyrfs*4) [31], a gene associated with xlRP. This mutation completely segregated in all 25 members from family RP-0858: it was present in eight cases, including six symptomatic and two asymptomatic female carriers and absent in the 17 unaffected members of the family. In RP-0911, a novel nucleotide change in *C1QTNF5* (NM_015645.3: c.489C>A), leading to the missense mutation p.Ser163Arg previously associated with Late-Onset Retinal Degeneration (LORD, #605670) [32] was identified in the index case. Validation and segregation of *C1QTNF5* p.Ser163Arg in the family revealed that, along with the five affected individuals, two of the family members initially considered unaffected also carried the mutation (S1 Fig, V:1 and IV:15). Table 3 summarizes causal mutations identified in the four families characterized.

Table 2. Clinical features of the four families genetically characterized in the present study. All the ages are expressed in years. adRP = autosomal dominant retinitis pigmentosa, DOB = date of birth, BE = both eyes, ERG = Electroretinogram, HM = High myopia, LE = left eye, LORD = Late Onset Retinal Dystrophy, LP = Light perception, MA = myopic astigmatism, MD = macular degeneration, MM = myopic maculopathy, NA = not available, NB = night blindness, NR = Non recordable, RE = right eye, RP = retinitis pigmentosa; RPE = Retinal pigment epithelium, VA = visual acuity, VEP = visual evoked potentials, VF = visual field, xlRP = X-linked retinitis pigmentosa.

Family (Gene)	Subject (DOB)	Revised diagnosis	Age at diagnosis	Age at onset NB/VF/VA	Age at time of testing	Visual field	Eye fundus	ERG	Visual acuity RE/LE	Other
RP-0107	III:2 (1915)	adRP	NA	12/40/35	NA	NA	NA	NA	NA	Myopia (18y) and cataract
(PRPH2)	III:6 (1924)	adRP	14	12/14/62	63	Absolute scotoma	Typical RP with macular alteration	NA	NA	Cataract (55y)
	IV:3 (1950)	adRP	NA	40/40/40	NA	NA	Salt-and-pepper pigmentation	NA	NA	
	V:3 (1976)	adRP	NA	NA	NA	NA	Salt-and-pepper pigmentation	NA	NA	
	V:4 (1980)	adRP	17	13/16/20	17	Diffuse relative scotoma	Salt-and-pepper pigmentation	NA	0.7/0.8	Dyschromatopsia
RP-0777	II:4 (1966)	adRP	NA	NA	32	Peripheral constriction	Typical RP, no macular affectation in BE	Reduced amplitudes typical of bilateral retinopathy	1.0/1.0	
(PRPF31)	III:1 (1934)	adRP	32	27/32/NA	79	Tubular field	Normal vessels and papilla, peripapillar atrophy 360°. No pigmentary lessions in BE	Rods: minimum reduced amplitude and increment of latencies; mix: minimum reduced amplitude in a wave and increment latencies in a and b waves; cones: minimum reduced amplitude b wave and minimum increment latencies in waves; flicker: normal in BE	0.7/0.8	
RP-0858	III:4 (1947)	xlRP	43	08/08/08	44	Concentric narrowing	Pale papilla, attenuated retinal vessels, peripheral pigment deposits in BE	NR BE	FC/LP	HM and PSC and altered VEP in BE (56y)
(RPGR)	III:6 (1943)	xlRP	NA	NA		NA	NA	NA	NA	Glaucoma
	IV:6 (1965)	xlRP	31	3/23/3	31	Severe concentric narrowing in BE	Typical RP	NR BE	FC/0.4	Strabismus, amblyopia RE, and myopia LE

(*Continued*)

Table 2. (*Continued*)

Family (Gene)	Subject (DOB)	Revised diagnosis	Age at diagnosis	Age at onset NB/VF/VA	Age at time of testing	Visual field	Eye fundus	ERG	Visual acuity RE/LE	Other
	IV:8 (1966)	xlRP	37	NA	45	Severe concentric narrowing in BE	Typical RP	NA	0.2/0.1	MM (BE)
	IV:9 (1969)	xlRP	35	Childhood	37	Severe concentric narrowing in BE	Pale papilla, attenuated retinal vessels, macular affectation in BE	Diffuse and severe impairment but not abolished	0.15/0.15	HM, MA, MM
	IV:10 (1974)	xlRP	35	NA	NA	NA	NA	NA	0.8/0.8	HM, MM (BE)
	V:2 (1998)	xlRP	14	NA	NA	Severely affected	Typical RP	NA	0.5/0.5	MA
	V:3 (1996)	xlRP	14	NA	14	NA	NA	NA	NA	NA
RP-0911	III:7 (1925)	LORD	60	43/43/40	67	NA	Macular atrophy and bone spicules in periphery	NA	0.5/0.2	Cataract (67y)
(*C1QTNF5*)	IV:1 (1946)	LORD	54	54/54/No	66	Central scotome	Normal papilla, RPE macular atrophy, no pigment	Rods and cones: abnormal amplitudes	0.8/0.8	
	IV:6 (1946)	LORD	NA	61/61/61	67	Central scotome nasal superior	RPE macular atrophy and hipopigmentary rounded areas	Rods: NR, mix: very reduced amplitudes cones and flicker: reduced amplitudes BE	0.1/0.4	Cataract (63y)
	IV:10 (1952)	LORD	59	59/No/No	62	Normal	Macular drusen	Normal	0.9/1	
	IV:11 (1956)	LORD	60	60/60/60	60	Central scotome	RPE macular atrophy, bone spicules in periphery	Rods: very reduced amplitude in b wave; mix and cones: reduced amplitude in a and b waves; cones: reduced amplitude in a and b waves, flicker: reduced amplitude in b wave BE	0.5/0.2	Cataract (56y)
	V:1 (NA)	LORD	NA	NA	NA	NA	NA	NA	NA	NA

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Table 3. Mutations detected in the four families included in the study. adRP: autosomal dominant Retinitis Pigmentosa; AMD: age-related macular degeneration; adCRD: autosomal dominant cone-rod dystrophy; LORD: Late-onset retinal degeneration.

Family ID	Suspected diagnosis	Final diagnosis	Mutation	Reference
RP-0107	adRP	adCRD	*PRPH2 c556G>A; p.Asp186Asn (NM_000322.4)*	Kohl *et al*. 2012 [33]
RP-0777	adRP	adRP	*PRPF31 del 54,619,134 to 54,634,248 (NM_015629.3)*	This study
RP-0858	adRP + high myopia	xlRP	*RPGR c.485_486delTT; p.Phe162Tyrfs*4 (NM_000328.2)*	Sharon *et al*. 2000 [31]
RP-0911	adRP + AMD	LORD	*C1QTNF5 c. 489C>A; p.Ser163Arg (NM_015645.3)*	Hayward C *et al*. 2003 [32]

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A novel large deletion spanning the entire *PRPF31* gene was identified in family RP-0777

The presence of causative CNVs was investigated in the 3 families not characterized by SNP/*indel* analysis with the XHMM algorithm: RP-0502, RP-0777, and RP-1405 using 663 individuals from 212 families as controls. A heterozygous deletion in the region chr19: 54,600,186–54,628,017 (Fig 1) was identified in the two affected members of family RP-0777 with XHMM. The region identified by this prediction tool included the first exon of the gene *OSCAR*, the entire sequence of *NDUFA3* and *TFPT* and exons 1 to 7 of *PRPF31* (Fig 1).

For validation of the CNV predicted by XHMM in *PRPF31*, we used a TaqMan pre-designed probe in exon 1 and five UPL probes spanning the entire gene region: exon 1, and introns 3, 7, 8 and 13 (Table 2). Both CNV assays with pre-designed TaqMan and UPL probes confirmed that the coding sequence of *PRPF31* from chr19:54,618,875 to chr19:54,634,248 (exons 1 to 13) was hemizygously deleted in the two affected and present at two copies in the six unaffected members of the family. The phenotype of family RP-0777 is summarized in Table 2.

No CNV was identified in families RP-0502 and RP-1405.

Screening of ORF15 in family RP-0502

In view of the results observed in family RP-0858 and due to the absence of male-to-male transmission in family RP-0502, we decided to screen this family for an X-linked inheritance model. Since *RPGR* was negative in this family (both by WES and gene-panels) and a large proportion of causal mutations of *RPGR* occur in the 3' end of the *ORF15* coding sequence, which was poorly covered in those sequencing assays, Sanger sequencing was used to scan for mutations in this gene. However, no mutation was found and therefore this family and family RP-1405, remain uncharacterized at the molecular level.

Discussion

In the present study, we applied WES to six families with an initial diagnosis of adRP that had been extensively screened for known causative mutations and/or genes. Using this approach, we were able to characterize four out of the six families and, although the four families carried mutations in known genes, the identification of the genetic defect by WES led to the reappraisal of the phenotype from the initial adRP to xlRP in RP-0858, to LORD in RP-0911 and cone-rode dystrophy in RP-0107. This allowed the establishment of a correct diagnosis, estimation of risk recurrence and genetic counseling in these families. adRP was initially considered the most plausible phenotype based on the mode of inheritance in families, patients' report on onset of symptoms such as night blindness, or visual acuity loss, and the ophthalmological data regarding fundus and visual field assessments. Clinical information was limited in some cases or exclusively recorded at later stages of the disease. This limitation, which is a common situation when studying this type of diseases, along with the clinical overlap of symptoms in

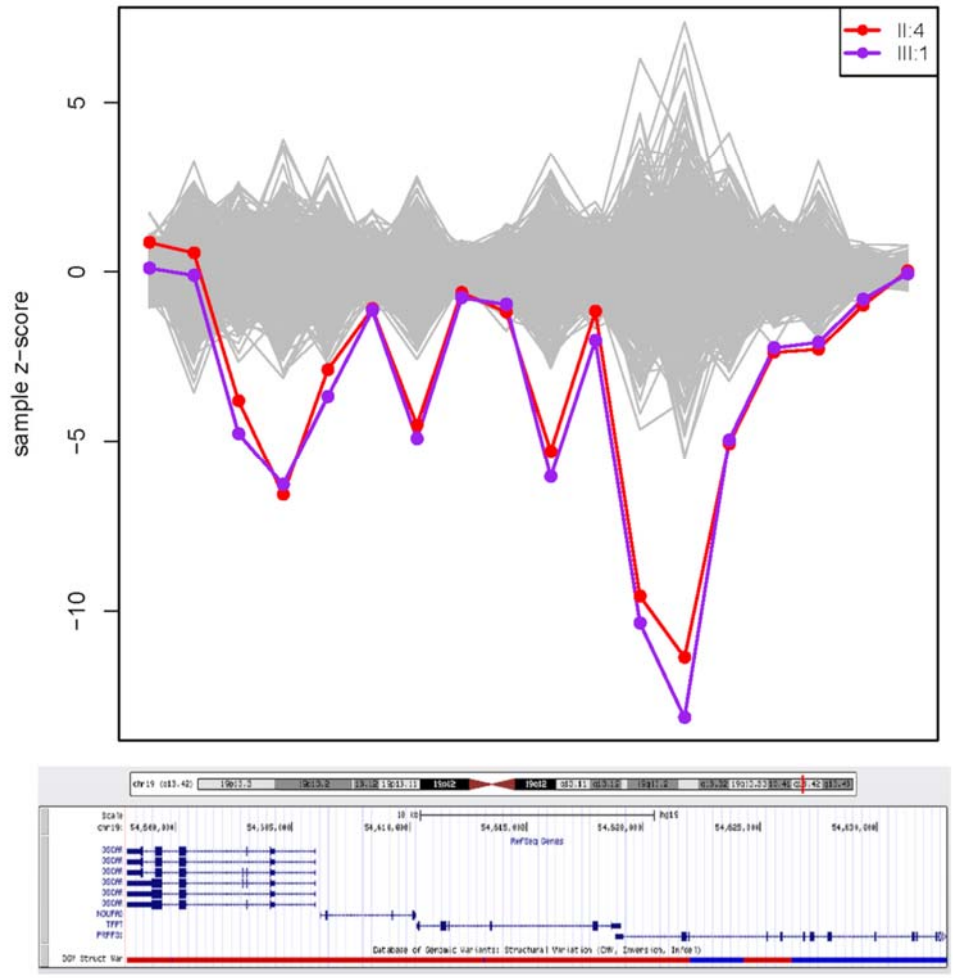

Fig 1. Region predicted by XHMM to harbor a heterozygous deletion. The x-axis represents the genome locus, with the genes and exons included in the algorithm, and the y-axis is the computed Z-score of PCA normalized read depth where positive values indicate predicted duplication and negative values deletion. The two affected individuals from family RP-0777 are highlighted with colors red (member II:4) and purple (member III:1) while grey lines represent the 663 control individuals used. Each point indicates a region containing an exon and they are paired with the corresponding exon/gene in a display of the region from the UCSC Genome Browser (http://genome.ucsc.edu/cgi-bin/hgGateway). On the left side of the UCSC Genome Browser track, the genes involved in the deletion are represented. From left to right, the genes/exons illustrated are exons 5 to 1 of the *OSCAR* gene, the entire genes *NUDFA3* and *TFPT* (4 and 6 exons, respectively), and exons 1 to 12 of *PRPF31*.

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different forms of inherited retinal dystrophies made establishing the precise diagnosis extremely complex and explains the reclassification of the phenotype in the families upon identification of the genetic cause. Therefore the use of a hypothesis free approach for mutation detection, such as WES, helps minimizing the impact that the availability of patient or family information has on the diagnostic success of retinal dystrophies.

Obligate carrier females of mutations in *RPGR* may display manifestations of the disease [8, 34], [35] and even be as severely affected as males [35, 36], as observed in family RP-0858, with all carrier females displaying a severe RP phenotype. What was remarkable in this family is that females affected largely outnumbered affected males (seven females versus three males). Additionally, the onset of symptoms was similar among both female and male carriers and there was no significant intra-familial variability in the symptomatology. These features are not often seen in xlRP and were along with the highly penetrant phenotype females presented the reasons why a dominant rather than an X-linked model was initially considered. Also, although myopia has been associated with xlRP caused by mutations in both *RPGR* and *RP2* [37–40], it is not exclusively found in this phenotype [41], and therefore was not used as a diagnostic criterion of xlRP. These results are consistent with the underestimated frequency of xlRP previously reported [35] and highlight the need of reviewing all adRP families with no male-to-male transmission, as already reported in [35], regardless of the severity of the symptoms, for testing of X-linked genes.

Family RP-0911 carried the mutation p.Ser163Arg *C1QTNF5* responsible for LORD, a very rare fully penetrant autosomal dominant retinal dystrophy with symptoms overlapping with a number of hereditary retinal conditions [42]. The phenotype of LORD evolves with time and in early stages can be misdiagnosed as early age-related macular degeneration (AMD), and later on with RP [42, 43], [44],[45], [46]. Due to the limited clinical data available in this family and the lack of literature about this entity by the time this family was first evaluated, LORD was never considered and different diagnoses were attributed to the three affected members from RP-0911: RP in IV:1, age-related macular degeneration in IV:6, and both in IV:11, initially suspecting of two distinct entities co-segregating in the family. Further clinical re-evaluation of the family evidenced that some of the symptoms were consistent with what has been reported in families affected with LORD whereas others, like neovascularization, was not present in any member of the family at the time of diagnosis. Interestingly, member IV:10, who had referred night blindness as the only symptom when she was 59 years, had evidence of drusen in the eye fundus at time of testing (62 years), but the rest of the ophthalmological study was completely normal. Unfortunately, we do not have information on the progression of the phenotype in this family, so a detailed description of the symptomatology and progression of the disease cannot be provided in this study.

WES also helped in the correct diagnosis of family RP-0107, who was found to harbor a mutation in *PRPH2* previously identified causing autosomal dominant cone dystrophy [30]. All affected members from family RP-0107 presented eye fundus and visual fields compatible with RP plus macular affectation, except for individual V:4 that had a diffuse retinal degeneration. Because the family was studied in the latest stages of the disease, the phenotype at that time was more compatible with RP than cone-rode dystrophy. Remarkably, in our diagnosis algorithm of adRP, *PRPH2* is one of the first genes to be screened, however in the index case of this family this was done by DGGE and possibly due to the sensitivity of the methodology, the mutation was not detected.

A large deletion spanning the entire region of *PRPF31* was detected in family RP-0777 using exome data. *PRPF31* is one of the most frequently mutated genes in adRP, accounting for 5–10% of the cases and with large deletions being responsible for almost 3% of the cases [34]. This deletion found in this study is similar to that reported by Kohn et al. [47] that besides *PRPF31* also included the genes *OSCAR*, *NUDFA3*, and *TFPT*. However, they found the breaking point of the

CNV in intron 11 and in our family, the deletion expands up to intron 13, at least, as evidenced by experimental validation with the UPL assays. The phenotype found in RP-0777, summarized in Table 3, is very similar to that reported by Kohn and colleagues [47] and surprisingly mild given the size of *PRPF31* deleted. Interestingly, despite NGS being widely used in search for mutations in retinal dystrophies, so far only Eisenberger *et al.* [48] and Nishiguchi *et al.* [49] have reported the discovery of large deletions in genes causing retinal dystrophies using sequencing data. With this study we support the feasibility of detecting CNV in genes responsible for retinal dystrophy using NGS, thus expanding the potential of this tool in the diagnosis of these diseases.

To our knowledge, WES has not been applied systematically to date as diagnostic tool in dominant forms of retinal dystrophies. This study was part of a larger multi-center sequencing effort on Mendelian disorders where WES has been successful identifying the genetic cause of a number of phenotypes, including the discovery of a new genetic cause of a syndromic form of RP in one of our Spanish families [50]. However, the results from the current study, with only known genes identified, indicate that WES may be an adequate and efficient tool once all known genetic causes of retinal dystrophy have been ruled out. For that purpose, targeted sequencing is regarded now as the most optimal approach of candidate gene screening [7, 10, 15, 51]. Almost simultaneous to this project, our group developed an NGS custom panel with 73 genes related to retinal dystrophies that was applied to 59 index cases of families with adRP, including the non-characterized families RP-0502 and RP-1405 [10]. The authors found a detection rate of 27% with 64% of the cases carrying new mutations in known genes, which is in line with previous results of studies performed in similar conditions [7], [8]. Very recently, Consugar et al. published a comparative analysis on the performance of panel-based versus WES [15]. The authors concluded that targeted sequencing was more sensitive for variant detection than WES, with a superior and more even coverage of genes, and therefore the preferred method for genetic diagnostic testing [15].

Based on our experience over the past years on the use of NGS technologies and in line with previous reports [7, 15, 51], we propose to start the diagnostic testing with targeted sequencing of candidate genes, due to the methodological and cost advantages over WES. As a second step, due to the high incidence of xlRP among families initially classified as dominant ([35] and this study), we propose to screen *ORF15* in any family showing no male-to-male transmission, regardless of the symptomatology or the number of females affected, to rule out a possible X-linked inheritance. *ORF15* is responsible for up to 60% of xlRP disease-causing mutations [52], and because of its high repetitive nature is not usually adequately covered by NGS methods. Finally, once known causes of inherited retinal dystrophies have been ruled out either WES or WGS may be used in the almost 50% of remaining cases that are estimated to harbor mutations in rarer novel genes [3].

Supporting Information

S1 Fig. Pedigrees of the six families studied. + and +/+: wild type genotypes. m: mutation detected in hemizygosis; m/+: mutation detected in heterozygosis. Filled and unfilled symbols represent affected and unaffected individuals respectively. Squares indicate males and circles females. Arrows indicate the index cases. Red circles represent individuals subjected to whole exome sequencing.
(TIFF)

S2 Fig. Eye fundus images from members of families RP-0777 and RP-0911. Each patient presents two fundus images per eye. A) Family RP-0777 and B) Family RP-0911. RE = Right eye. LE = Left eye.
(TIFF)

S3 Fig. Electroretinogram recordings from members of families RP-0777 and RP-0911.
(TIFF)

S1 Table. Genetic screening performed to the six families prior to whole exome sequencing. **SSCP:** Single Strand Conformation Polymorphism; **DGGE:** CG-clamped Denaturing Gradient Gel Electrophoresis. The parentheses indicate the exons targeted by these techniques; otherwise the entire gene was screened. For ADRP Chip, version 1 includes 355 SNPs in *CA4*, *CRX*, *FSCN2*, *IMPDH1*, *NR2E3*, *NRL*, *PRPF3*, *PRPF31*, *PRPF8*, *PRPH2*, *RHO*, *ROM1*, *RP1*, *RP9*, *TOPORS*; and version 2 includes 414 SNPs in *CA4, CRX, FSCN2, IMPDH1, KLHL7, NR2E3, NRL, PRPF3, PRPF31, PRPF8, PRPH2, RHO, ROM1, RP1, RP9, TOPORS; Sanger sequencing was used to screen mutations in exons 16 and 25 for SNRNP200, exon 2 for NR2E3 and exon 13 for GUCY2D. For IMPDH1 all exons were sequenced. RD_NGS_Panel refers to the custom Next Generation Sequencing panel from* S2 Table.
(DOCX)

S2 Table. Genes associated with RP and LCA included in the customized RD_NGS_Panel. Macular dystrophy (MD); retinitis pigmentosa (RP); Leber's congenital amaurosis (LCA); congenital stationary night blindness (CSNB); choroideremia (CHM), cone-rod dystrophy (CORD); autosomal recessive (ar); autosomal dominant (ad); X-linked (xl); McKusick-Kaufman syndrome (MKKS); Senior Loken Syndrome (SLS); vitreoretinopathy proliferative (VRP); enhanced S-cone syndrome (ESC).
(DOCX)

S3 Table. Primers and conditions used for PCR and sequencing of *ORF15*.
(DOCX)

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Author Contributions

Conceived and designed the experiments: BA PFS MM RP YG LT XX HH CA. Performed the experiments: PFS MM RP XL LG JZ. Analyzed the data: BA JL PFS YL MM RP RG MC YG LT. Contributed reagents/materials/analysis tools: JL XL LG JZ XX. Wrote the paper: BA PFS YL MM RP MC YG LT BK HH CA. Clinical evaluation of the patients and the interpretation of the phenotypic analysis: FBK MILM BGS.

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Additional files

Additional file 1: Clinical data of family RP-0482 and primers used in the study.

Clinical evaluation of family RP-0482

Diagnostic evaluations of patients were based on ophthalmological, neurological and otological examinations. Ophthalmological evaluation included the assessment of visual acuity, intraocular pressure, ocular motility, pupillary reaction, biomicroscopic slit-lamp examination, dilated fundus examination, best-corrected visual acuity, visual-field tests, fundoscopy, autofluoresce, optical coherence tomography examinations and full-field and multifocal electroretinogram (ERG) responses. Visual function was performed by static perimetry, D15 panel testing and Ganzfeld electroretinography according to the International Society for Clinical Electrophysiology of Vision Standards [1] with a UTAS 2000 system (LKC Technologies, Gaithersburg, USA) and jet electrodes.

Diagnosis of RP was based on clinical evaluation of night blindness, progressive visual field constriction, and poor visual acuity, and confirmed by altered or abolished ERG responses. Neurological evaluation included examination of cognitive status, cranial nerves, deep tendon reflexes, sensory and motor systems evaluations, and cerebellar testing. MRI was also performed and hearing assessment was done by pure tone audiometry and otoscopic examination.

Clinical data

Proband (IV:3)

History: Patient IV:3 suffered from congenital horizontal nystagmus in both eyes. Her parents reported her first walking at the age of two and having gait abnormalities since then. At the age of four, she started experiencing night blindness and loss of VA and she was first diagnosed with optic atrophy (OA)when she was five and with RP at 14. The patient was first studied in our hospital at 19 years of age. Ophthalmological examination at this age showed constriction of visual field to central 20º and loss of VA to 20/200 in both eyes. Both scotopic and photopic ERG responses were not recordable above noise and the visual evoked potentials (VEP) responses were delayed. Posterior subcapsular cataract was found in both eyes. Fundus showed pale and atrophic papilla, narrowed vessels, bone-like spicule pigmentations and

retinal pigment epithelium (RPE) atrophy at the mid periphery of both eyes, and a well-defined macular lesion in the right eye. Neurological examination showed moderate psychomotor retardation, mild intellectual disability, loss of strength, *pes cavus* and bilateral and symmetrical cerebellar alteration. Biopsy of skeletal muscle showed the presence of striated muscle tissue with signs of re-innervation. Head MRI at 20 years did not show any significant alteration. Audiometry testing evidenced asymmetric sensorineural hypoacusis in both ears (90Dbs right ear and 30Dbs left ear) that currently has progressed to need hearing aid.

Examination at 34 years: Over the last 15 years, she has shown a slow progressive VA loss to hand motion in the right eye and to < 20/400 in the left eye. The macular atrophy has also progressed in both eyes as revealed by OCT, funduscopy and autofluorence images (Figure 2A-D). Her latest neurological evaluation evidenced symmetrical Charcot-Marie-Tooth-like sensorimotor polyneuropathy with cranial nerves unaltered, biceps and triceps reflex altered and radius, knee and ankle reflexes abolished. The motor evaluation showed muscle strength altered in distal lower limb and in proximal upper limbs. She also displayed vibratory, touch and algesic distal sensory loss, *pes cavus* and atrophy in both hands. Cerebellar examination evidenced dysmetria, ataxia and intention tremor and head MRI confirmed moderate cerebellar atrophy.

Affected sister (IV:2)

History: Patient IV:2 has no history of psychomotor or intellectual delay. She complained of slightly poor night vision at the age of 16 and OA was suspected due to altered responses on VEP. She was first studied when she was 23 years of age, when an ophthalmological exam evidenced both eyes exhibiting pale optic disc, slightly attenuated retinal vessels, bone spicules at the extreme mid-periphery and conserved macula. She also showed peripheral scotomes in the right eye, however, she had normal ERG responses and VA was not reduced (20/20). Auditory and neurological examinations were also normal.

Examination at the age of 36: The patient complained of photophobia and photopsias but showed no loss of peripheral vision and/or VA. Funduscopy evidenced only minor changes compared to the first exams, including a slight RPE atrophy. Full-field ERG exhibited normal scotopic and photopic responses but multifocal ERG revealed significantly lower responses at perifoveal regions with foveal preservation. OCT images confirmed an atrophy of the photoreceptors layer in the perifoveal region and the normal foveal thickness. Neurological evaluation was normal and head MRI did not show cerebellar atrophy.

Affected mother (III:2)

History: Patient III:2 complained of night blindness, loss of VA and constrained visual field since childhood but she was not diagnosed with RP until 47 years of age. At this age, she presented loss of VA (20/200, right eye and 20/25, left eye). The VEP responses were abolished in the right eye and very altered in the left. She was diagnosed with an asymmetric sensorineural hearing loss (40Dbs in left ear and normal in right ear) at 50 years. The patient was first studied in our hospital at 55 years of age. Ophthalmological examination at this age showed constriction of visual field and progressive loss of VA to counting fingers in the right eye and to 20/50 in the left eye. Scotopic ERG responses were not recordable and photopic responses were very diminished. Fundus showed pale and atrophic papilla, narrowed vessels and sparse spicule pigmentations in both eyes. Right eye also manifested patches of atrophy at the mid periphery and macular regions.

Examination at 70 years: The patient's latest ophthalmological examination showed a VA of counting fingers in the REand of 20/400 in the LE, and absence of scotopic and photopic ERG responses. Funduscopy reflected a very pale and atrophic papilla, narrowed vessels, choriocapillar atrophy and sparse spicule pigmentations in both eyes. The right eye displayed a very well defined macular lesion of atrophy and the left presented an alteration in RPE. Neurological examination evidenced asymmetrical sensorimotor polyneuropathy, with slight loss of strength, and mild bilateral thenar muscle atrophy. The sensory evaluation demonstrated a deep sensory loss. Cerebellar exploration revealed mild intention tremor and ataxia (8.5 in the Scale for the Assessment and Rating of Ataxia -SARA-), a slight dysmetria, predominant in left upper and lower limbs, and *pes cavus*. Head MRI exhibited mild cerebellar atrophy.

Previous genetic screenings in the family

Genomic DNA samples were extracted from peripheral blood lymphocytes from all participating individuals of the family and controls (BioRobot EZ1 Qiagen, Hilden, Germany). Additionally, DNA from saliva (Oragene containers, DNA Genotek), and total RNA from peripheral blood (PAXgene blood RNA kit, Qiagen) were extracted from individuals III:2, IV:1, IV:2 and IV:3.

Known mutations in autosomal dominant RP (adRP) genes were previously excluded in the index case using a genotyping microarray based on Arrayed Primer Extension (APEX) technology (adRP chip, Asper Ophthalmics, Tartu, Estonia). Differential diagnosis was performed with other pathologies with overlapping symptoms such as mithocondriopathies, ataxic forms of RP and Refsum disease. Mitochondrial mutations in *MTTH*, *MTTK*, *MTTS2*, and *MTTL2* and those associated with NARP (Neurogenic muscle weakness, Ataxia, and Retinitis Pigmentosa, MIM 551500) and Leigh Syndrome (MIM 256000) were discarded using Sanger sequencing. Screening for mutations in *SCA7*, associated with a form of ataxia with RP, spinocerebellar ataxia type 7 (MIM 164500), and peroxisomal function testing for Refsum disease (MIM 266500) were negative. Karyotype of the proband was also normal.

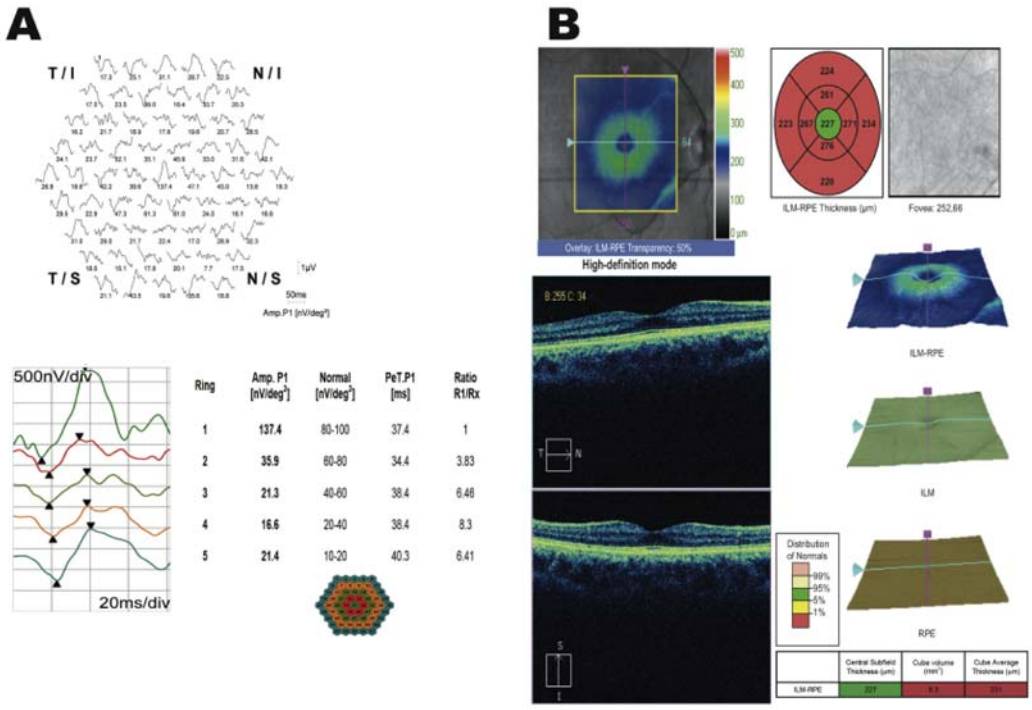
Primer sequences used in the study

Primers used for validation and segregation of the candidate mutations in family RP-0482

Position	Gene	Aminoacid change	Reference Sequence	Sequence_Primer_F (5'>3')	Sequence_Primer_R (5'>3')
chr7:48312363	ABCA13	p.L1034I	NM_152701	GGCTCTTCGTTGACTTTCCTT	CCAGTTCTTGGCCCTCTGTA
chr16:3554786	CLUAP1	p.P30R	NM_015041	TGTGATGGGGAACTGAATGA	ACTTTCCCTTTTGCAGCATC
chr13:99099055	FARP1	p.E1014K	NM_005766	GCTCACCATCCCCTCTGAGT	ACTCGCTGACTTGGGAGAAA
chr8:11687784	FDFT1	p.D78G	NM_004462	CTATGCACACGCTGACCTGT	CCCTTCCCTACCTGTGGAAT
chr1:203033044	PPFIA4	p.M24T	NM_015053	CTTCCTCCCCCATAATAGCC	GAGGCAGACCCTCAGAGCTA
chrX:106871904	PRPS1	p.S16P	NM_002764	ATTGAGTCTGTGGCCGACTT	GGGCAGGTGAGGTCTAGTCA
chr3:51696477	RAD54L2	p.G475C	NM_015106	TTTGGGGACTACAGGACAGG	ATGATCTCTGGGCTGTCAGG
chr1:110883886	RBM15	p.G620A	NM_022768	TCGGACTGCAGCTACTTCTG	ATCCAGATGACGTCCTCCAC
chr3:50112732	RBM6	p.Y414C	NM_005777	GATGGTGTCTGCTTCTTTTGC	GGCAAAGACATCACTCTAGGC
chr1:84963141	RPF1	p.K346N	NM_025065	AACCCGGTTTTTCAGCACTA	ATTCGTCCAGACGTGAAAGG
chr18:74617295	ZNF236	p.R739C	NM_007345	GGAAGAGGCTTTGTTTCTGC	CACCAGAATTACACCCACCTG
chr2:135975092	ZRANB3	p.P811R	NM_032143	TCTGCCTGTGAGCTGTTAACTC	CCCCTGTAGAAAGAACCATGA

Additional file 2.

Figure S1. Multifocal ERG and Optical coherencetomography (OCT) findings suggested a sectorial affectation of perifovealphotoreceptors. **A.** Right eye multifocal ERG (mfERG) records in patientIV:2 demonstrate a reduced function of photoreceptors of perifovealregion with preserved function of fovea. The ring analysis (see schematic)goes from the centre to the periphery. Quantitative results of mfERGanalyses are displayed in table format. **B**. OCT macular cube 512Å~128scan show macular thickness in the right eye from patient IV:2. ILM: innerlimiting membrane; RPE: retinal pigment epithelium. Top left: fundusimage with scan cube overlay. Top right: macular thickness significancemap. The central innermost 1-mm-diameter circle represents the centralsubfield; inner superior, inner nasal, inner inferior, and inner temporalareas bounded by the 3-mm-diameter circle form the inner macula; outersuperior, outer nasal, outer inferior, and outer temporal areas bounded bythe 6-mm-diameter circle form the outer macula. Retinal thickness valuesfrom ILM to RPE are compared to the normative data. Middle and bottomleft: cross-sectional OCT scans. Middle right: 3days surface maps: theILM-RPE, displaying the retinal thickness in three dimensions. Bottomright: central subfield thickness, overall average macular thickness, andoverall macular volume compared to normative data are displayed intable format. Reduction in the retinal thickness in the perifoveal región with a normal foveal thickness is also evidenced.



Additional file 3:

Table S1 and S2. Genetic variants obtained (single nucleotide variants –SNV-, **Table S1**, and insertion deletion variants –indels-, **Table S2**) after the first variant filtering analysis.

Table S1

Chr	Position	rsid	1KGP	ESP	AA chan	Gene	GERP++	PhyloP	LRT	Poly	SIFT	MutationTaster
1	12089299				p.R226C	*MIIP*	2.33	C	N	PrD	T	N
1	27240176				p.R86W	*NR0B2*	2.78	C	N	PsD	D	N
1	52954676				p.D474N	*ZCCHC11*	5.04	C	D	PrD	T	N
1	84963141				p.K346N	*RPF1*	5.61	C	D	PsD	D	D
1	110883886				p.G620A	*RBM15*	4.42	C	D	B	T	D
1	117663358	rs139632476	0.0005	0.0008	p.H156Y	*TRIM45*	4.65	C	N	PrD	T	N
1	151739750				p.H48R	*OAZ3*						
1	152659479	rs148459371	0.0005	0.0005	p.G54S	*LCE2B*	-0.91	NC	NA	NA	D	N
1	154316895		0.0005		p.S720F	*ATP8B2*	5.3	C	D	PrD	D	D
1	155221575			0.0001	p.P14L	*FAM189B*	4.48	C	D	PrD	D	N
1	155233070				p.A252V	*CLK2*	4.97	C	N	B	T	N
1	156756783				p.E300D	*PRCC*	1.97	NC	N	B	T	D
1	158262505				p.Q244K	*CD1C*	-0.353	NC	N	B	T	N
1	172571942			0.0001	p.I1094L	*C1orf9*						
1	186092095			0.0001	p.I4081T	*HMCN1*	5.52	C	D	PrD	D	D
1	203033044				p.M24T	*PPFIA4*						
1	219366546	rs145589411	0.0005	0.0012	p.I105T	*LYPLAL1*	5.48	C	D	PrD	D	D
1	229730691	rs41304137	0.0009	0.0018	p.T375A	*TAF5L*	4.6	C	D	PsD	D	D
1	245861438			0.0001	p.L1952P	*KIF26B*						
1	247614909				p.R126C	*OR2B11*	3.3	C	N	PrD	D	N
2	75882376	rs189220165	0.0005		p.I282V	*MRPL19*	2.21	NC	D	B	T	N
2	133542717			0.0003	p.T556M	*NCKAP5*						
2	135621175				p.A154T	*ACMSD*	1.34	C	D	B	T	D
2	135975092				p.P811R	*ZRANB3*						
2	160697271	rs147904044	0.0023	0.0041	p.S1159F	*LY75,LY75-CD302*	3.11	C	NA	PrD	T	NA
2	160735174	rs147820690	0.0014	0.0022	p.G525E	*LY75,LY75-CD302*	4.37	C	N	PrD	D	NA
2	171687495			0.0001	p.V114M	*GAD1*	5.55	C	D	B	T	N
2	217001820	rs192418356	0.0005		p.V261F	*XRCC5*	3.95	NC	D	PrD	D	D
3	49940430			0.0001	p.A205T	*MST1R*	-8.91	NC	N	B	T	N

Chr	Position	rsid	1KGP	ESP	AA change	Gene	GERP++	PhyloP	LRT	Poly	SIFT	MutationTaster
3	50112732				p.Y414C	*RBM6*	3.34	C	N	PrD	D	D
3	51696477				p.G475C	*RAD54L2*	4.93	C	D	PrD	D	D
3	75714929	rs148715408			p.A196T	*FRG2C*	-1.33	NC	N	PsD	T	N
3	75714950	rs147607693			p.L203M	*FRG2C*	0.8	NC	N	B	T	N
3	120352045				p.E238D	*HGD*	0.531	NC	D	B	T	D
3	167245750	rs147197491	0.0005	0.0002	p.R294I	*WDR49*	3.4	NC	N	PrD	T	N
3	195452987	rs9866681			p.A299T	*MUC20*						
3	195452991	rs3828411			p.T300I	*MUC20*						
4	48993551				p.V106L	*CWH43*	2.72	C	N	B	T	D
4	106158350	rs75056899	0.0027	0.0026	p.Q1084P	*TET2*	0.37	C	N	PsD	D	N
4	147755012	rs41280533		0.0014	p.D308V	*TTC29*						
4	148559898	rs76148346	0	0.0008	p.V419I	*PRMT10:PRMT10*	4.85	C	D	PsD	D	N
5	149301253	rs114973968	0.0037	0.0036	p.P212L	*PDE6A*	5.06	C	D	PrD	D	D
5	176008381				p.R619H	*CDHR2*	-5.02	NC	NA	PrD	NA	N
5	179192490	rs141968004		0.0001	p.P160H	*MAML1*	4.59	C	D	PrD	T	D
6	31238909	rs1050686			p.T66K	*HLA-B,HLA-C*	-4.24	NC	U	B	T	NA
6	31238910	rs1050685			p.T66A	*HLA-B,HLA-C*	-4.24	NC	U	B	T	NA
6	56483679	rs45487998	0.0037	0.0023	p.K1718T	*DST*	2.75	NC	NA	NA	T	N
6	74528127	rs35238647	0.0018	0.0042	p.R1233G	*CD109*	2.47	NC	U	PsD	T	N
7	48312363				p.L1034I	*ABCA13*						
7	99084275	rs138157337	0.0005	0.0009	p.D90N	*ZNF789*	-1.74	NC	NA	B	T	N
7	131895706		0.0014	0.0002	p.T765I	*PLXNA4*	4.86	C	N	PsD	NA	D
7	138269570	rs34585297	0.0009	0.0023	p.R975S	*TRIM24*	3.2	C	N	B	T	N
8	11687784				p.D78G	*FDFT1*	5.57	C	D	PrD	D	D
8	37691254				p.V449M	*GPR124*	3.06	C	D	B	T	N
8	56723438					*TGS1*						
8	98041687				p.G340R	*PGCP*	5.29	C	D	B	T	D
8	146156974				p.T400N	*ZNF16*	3.55	C	NA	B	D	N
9	35704339	rs144809355	0.0009	0.0006	p.A2013T	*TLN1*	4.65	C	D	PsD	D	D
9	36249368				p.K26N	*GNE*						

Chr	Position	rsid	1KGP	ESP	AA change	Gene	GERP++	PhyloP	LRT	Poly	SIFT	MutationTaster
9	37304236	rs138176259	0.0014	0.0024	p.S236A	*ZCCHC7*	-2.19	NC	N	B	T	N
9	78808173	rs148131474		0.0008	p.E157K	*PCSK5*	5.2	C	NA	PrD	D	N
10	7763669	rs147906523		0.0004	p.E266Q	*ITIH2*	3.86	C	N	B	T	D
10	46999289	rs140859626	0.0027	0.0018	p.R137W	*GPRIN2*	1.69	C	N	PrD	D	N
10	98819233				p.R357C	*SLIT1*	4.44	C	D	PrD	D	D
10	102781975				p.Y237C	*PDZD7*	4.94	C	D	PrD	D	D
10	105793895	rs150282756	0.0018	0.0004	p.G1322S	*COL17A1*	4.1	C	N	NA	D	D
10	124914494				p.K21E	*BUB3*	2.98	NC	D	B	D	D
10	134646912				p.R2356Q	*TTC40*	3.49	C	D	NA	NA	N
11	5701205				p.R68Q	*TRIM5*	2.78	C	N	B	T	N
11	126126712	rs180953673	0.0009		p.R316Q	*FAM118B*	4.67	C	D	PrD	T	D
12	10954919	rs144677090		0.0003	p.I84T	*TAS2R7*	4.01	C	N	PsD	T	N
12	10978391				p.T160A	*TAS2R10*	-4.78	NC	N	B	T	N
12	118517228		0.0014	0.0012	p.S283L	*VSIG10*						
12	133277845				p.A137T	*PXMP2*	-10.3	NC	N	B	T	NA
13	39454452	rs114400765	0	0.0019	p.T3013M	*FREM2*	5.42	C	D	PrD	D	D
13	99099055				p.E1014K	*FARP1*	5.1	C	D	PrD	T	D
14	23745014		0.0018	0.002	p.Q475E	*HOMEZ*						
14	80997207			0.0002	p.H968Q	*CEP128*	5.57	C	D	PrD	T	N
14	93649990	rs35855685		0.0037	p.V200I	*MOAP1*	2.83	C	NA	B	T	N
14	107034967	rs72686844			p.K38R	*immunoglobulin heavy chain*						
15	22867569				p.R882H	*TUBGCP5*	3.9	C	D	PrD	D	D
15	40915719				p.M936T	*CASC5*	0.922	NC	N	PrD	T	N
15	76914159	rs184003295	0.0023	0.0038	p.Y194F	*SCAPER*						
15	86236607				p.I42V	*AKAP13*	1.73	NC	NA	B	T	N
15	91545322				p.V364L	*VPS33B*	2.89	NC	D	PsD	T	D
16	1400112	rs142109530	0.0041	0.0022	p.A217V	*C16orf42*	0.819	NC	N	B	T	N
16	2546790		0	0.0011	p.R214H	*TBC1D24*	5.08	C	D	PrD	NA	D
16	2979735				p.G17S	*FLYWCH1*						
16	3554786				p.P30R	*CLUAP1*	4.02	C	D	PrD	D	D

Table S2. Genetic variants obtained (insertion deletion variants –indels-) after the first variant filtering analysis.

Chr	Position	rsid	Reference allele	Alternate allele	AA change	Effect	Gene	Impact
1	14106394	rs148293494	A	ACTC	T501TP	Codon insertion	*PRDM2*	Moderate
1	109465165	rs111974813	ACTT	A	TS523T	Codon deletion	*GPSM2*	Moderate
1	152681680	rs11269814	C	CAGCTCTGGGGGCTGCTGT	-44SSGGCC	Codon insertion	*LCE4A*	Moderate
2	38179414	rs141540819	CTG	C		Frame shift	*FAM82A1*	High
3	97983487	rs11279406	TTGTAACCAC	T	LVTT120L	Codon deletion	*OR5H6*	Moderate
5	156479443	rs143959546	CTTG	C	TS200S	Codon change plus deletion	*HAVCR1*	Moderate
6	49437853	rs11333073	AT	A		Splice site acceptor	*CENPQ*	High
6	82461727		ACCGCCGAAGTCGCCG	A	GGDFGG120G	Codon change plus deletion	*FAM46A*	Moderate
9	107360768	rs11314210	GT	G		Frame shift	*OR13C5*	High
9	107367392	rs143198170	TGTTA	T		Frame shift	*OR13C2*	High
9	107367664	rs143760725	AGC	A		Frame shift	*OR13C2*	High
11	4790873	rs141621954	CG	C		Frame shift	*OR51F1*	High
11	48285981	rs112100800	CCTT	C	TF190T	Codon deletion	*OR4X1*	Moderate
17	38858134	rs11309872	CA	C		Frame shift	*KRT24*	High
17	39240781		CCTGCTGCCGCCCCAG	C		Frame shift	*KRTAP4-9*	High
19	49657710	rs10533934	ACAT	A	DV231V	Codon change plus deletion	*HRC*	Moderate
20	44520237	rs10582052	CCTG	C	L11-	Codon deletion	*CTSA*	Moderate
21	11029596	rs138714104	AC	A		Splice site donor	*TPTE*	High

Table S3. Variants yielded by the second filtering of the 141 variants based on the novelty, conservation across species according to the values from PhyloP and GERP++, predicted pathogenicity at least by two of the systems evaluated and expression in the retina. 1KGP: Minor allele frequency in the 1000 Genome Project, ESP: Minor allele frequency in the NHLBI GO Exome Sequencing Project. For PhyloP prediction, C: Conserved, NC: Not conserved. For pathogenicity prediction systems, B: benign, D: deleterious (LRT), or damaging (SIFT) or disease causing (MutationTaster), N: neutral (LRT) or polymorphism (MutationTaster), NA: missing data, PrD: probably damaging, PsD: possibly damaging, T: tolerated, U: Unknown.

Position	Aminoacid change	Gene	GERP ++	PhyloP	LRT	PolyPhen2	SIFT	Mutation Taster
chr7:48312363	p.L1034I	*ABCA13*		Conserved (UCSC)				
chr16:3554786	p.P30R	*CLUAP1*	4.02	C	D	PrD	D	D
chr13:99099055	p.E1014K	*FARP1*	5.1	C	D	PrD	T	D
chr8:11687784	p.D78G	*FDFT1*	5.57	C	D	PrD	D	D
chr1:203033044	p.M24T	*PPFIA4*		Conserved (UCSC)				
chrX:106871904	pS16P	*PRPS1*	4.1	C	D	B	D	D
chr3:51696477	p.G475C	*RAD54L2*	4.93	C	D	PrD	D	D
chr1:110883886	p.G620A	*RBM15*	4.42	C	D	B	T	D
chr3:50112732	p.Y414C	*RBM6*	3.34	C	N	PrD	D	D
chr1:84963141	p.K346N	*RPF1*	5.61	C	D	PsD	D	D
chr18:74617295	p.R739C	*ZNF236*	5.34	C	D	PrD	D	D
chr2:135975092	p.P811R	*ZRANB3*		Conserved (UCSC)				

CAPÍTULO VI

Discusión

Discusión

El objetivo principal de esta tesis ha sido profundizar en el conocimiento sobre las bases genéticas y moleculares de pacientes con adRP. Por ello hemos utilizado diferentes herramientas moleculares tanto clásicas como de secuenciación masiva, con el fin de identificar los genes y mutaciones responsables de la enfermedad y así poder determinar la implicación real de algunos de los genes más importantes asociados a adRP en población española, desarrollar un algoritmo de diagnóstico genético coste efectivo para este grupo de familias, establecer correlaciones genotipo-fenotipo que nos permitan orientar el diagnóstico e identificar nuevas mutaciones y genes asociados a DR. Estos estudios se aplicaron en 200 familias seleccionadas de nuestra cohorte de familias con DR, con un diagnóstico inicial de adRP, estudiadas por los servicios de Genética y Oftalmología del Hospital Universitario Fundación Jiménez Díaz de Madrid.

Implicación del gen *RHO* en adRP

En el primer trabajo de esta memoria se abordó el estudio clínico y molecular de las familias con adRP que presentaban mutaciones en el gen *RHO*. Desde que este gen fue identificado (Nathans *et al.,* 1986) y asociado a RP (Dryja *et al.,* 1991) se ha estudiado ampliamente por ser el más frecuentemente mutado en pacientes con adRP. El estudio retrospectivo realizado nos ha permitido resumir y revisar los datos moleculares y clínicos de pacientes con mutaciones en *RHO*, establecer su prevalencia en población española, además de describir correlaciones genotipo-fenotipo de las diferentes variantes asociadas y establecer un algoritmo diagnóstico preciso para estas familias.

En este estudio se han identificado mutaciones en RHO en el 21% (42/200) de nuestras familias con adRP, lo que confirma la tasa de detección de mutaciones descrita previamente en nuestra población (Millá *et al.,* 2002). Estos datos no son estadísticamente diferentes (p=0,66) a las frecuencias descritas para otras poblaciones europeas (alemana, italiana y francesa, respectivamente) de alrededor de un 16% (Bunge *et al.,* 1993; Ziviello *et al.,* 2005; Audo *et al.,* 2010). En otras poblaciones no europeas, esta tasa de detección varía entre el 6% y 28% en China e Israel, respectivamente (Li *et al.,* 2010; Beryozkin *et al.,* 2016).

En este trabajo se observó un amplio **espectro mutacional** detectándose un total de 27 variantes distintas en el gen *RHO.* El 26% (7/27) de los cambios identificados en estas familias no habían sido descritos previamente. Fueron clasificadas como variantes causantes de la enfermedad basándonos en la elevada conservación de los aminoácidos alterados en

distintos ortólogos de la proteína rodopsina, la predicción de su patogenicidad mediante programas *in silico*, su ausencia en más de 200 cromosomas de la población de control española y, cuando era posible, la cosegregación dentro de las familias. Un total de ocho mutaciones se detectaron en al menos dos familias (p.Thr58Arg, p. Gly106Arg, p.Arg135Trp, p.Ala164Glu, p.Cys167Tyr, p.Gly182Ser, p.Asp190Tyr y p. Pro347Leu). La mutación más frecuente fue p. Pro347Leu identificada en nueve de las familias representando así el 4,5% en nuestra cohorte de 200 pacientes índice de modo similar a lo descrito para otras poblaciones europeas (Fujiki *et al.,* 1992; Greenberg *et al.,* 1999; Ziviello *et al.,* 2005; Audo, *et al.,* 2010). Esta mutación está considerada como *hot spot* debido a que afecta a una secuencia CpG en la que se ha descrito un aumento de la probabilidad de transición C>T (Crow, 2000). En al menos dos familias se pudo descartar la existencia de un posible efecto fundador al detectarse *SNPs* (*Single Nucleotid Polymorphisms*) próximos a la mutación, rs373118114 y rs2071093 en las familias RP-0685 y RP-0900 respectivamente. Otra mutación frecuente en el gen *RHO* es p.Pro23His, presente en el 8,5% en población norte americana (Dryja *et al.,* 1990) que sin embargo, no ha sido identificada en nuestra cohorte española, ni tampoco se ha observado en otros trabajos europeos (Ziviello *et al.,* 2005; Audo *et al.,* 2010).

Dos tercios de las mutaciones identificadas en nuestro estudio se localizan en los exones 1 y 3, y en la región la región carboxi-terminal de la proteína (Figura S1). Esta distribución de las mutaciones es similar a lo descrito previamente en bases de datos (http://www.retina-international.org). Todas las variantes encontradas fueron de tipo *missense*, a excepción de un cambio conocido en la región de *splicing* del exón 3 y una nueva variante *nonsense*, p. Trp126*, que fue detectada en el exón 2 de *RHO.* Estos resultados son concordantes con el tipo de mutaciones en *RHO* recopiladas en la base de datos HGMD. Aunque se ha descrito que el principal mecanismo etiopatogénico asociado a *RHO* es la dominancia negativa asociada a mutaciones de tipo *missense, in-frame y stop loss*, la identificación de variantes tipo *LOF* (como *frameshift, nonsense, splicing* y *CNVs*) evidencia que deben existir otros tipos de mecanismos implicados (Macke *et al.,* 1993; Eisenberger *et al.,* 2013). Este es el caso de la mutación Tyr136* previamente asociada a una forma muy leve de inicio tardío (Sánchez *et al.,* 1996) en la que se ha descrito un mecanismo de haploinsuficiencia debido a degradación del ARNm por el mecanismo *nonsense-mediated decay (NMD*) (Roman-Sanchez *et al.,* 2016). Un mecanismo similar podría ser el responsable del fenotipo leve asociado a nuestra mutación Trp126*. Aunque se ha descrito que las mutaciones *nonsense* en rodopsina serían alelos recesivos (Rosenfeld *et al.,* 1992; Kartasasmita *et al.,* 2011), únicamente dos de ellas se han asociado a arRP (Trp161* y Glu249*) mientras que en adRP se

han identificado al menos siete variantes *nonsense* más, con distinta severidad de fenotipos. Esta variabilidad fenotípica podría originarse por el distinto grado de toxicidad causado por las proteínas truncadas o por el efecto diferencial del *NMD* sobre la estabilidad del tránscrito conteniendo el codón de parada prematura (Roman-Sanchez *et al.,* 2016).

Algunos autores han sugerido que en el mecanismo molecular por el que las mutaciones de pérdida de función en *RHO* provocan degeneración retiniana está implicada la activación de la vía celular calpaína-Factor Inductor de Apoptosis (Aif); mientras que las mutaciones que provocan mal plegamiento de la proteína, además de activar esta vía Calpaína-Aif, se acumulan en el retículo endoplasmático (RE), lo que induce la activación de la respuesta de estrés del RE y con ello provoca un colapso de la homeostasis y degeneración retiniana. La implicación de estas vías supone una posible diana para futura terapia farmacológica (Comitato *et al.,* 2016). También, se han realizado estudios de terapia génica basados en la supresión y posterior reemplazo del gen *RHO*. Para ello se ha tratado de silenciar el gen mediante diferentes estrategias como la inyección de ARNs de interferencia (Gorbatyuk *et al.,* 2007; Chadderton *et al.,* 2009; Mao *et al.,* 2012) y más recientemente, otros represores trancripcionales de nueva generación, incluyendo TALEs y CRISPR-cas9 inactivos (Kabadi and Gersbach, 2014). Varios grupos han logrado suprimir la expresión de *RHO* con éxito a niveles terapéuticamente relevantes en modelos animales (O'Reilly *et al.,* 2007; Mussolino *et al.,* 2011; Mao et al. 2012; Farrar *et al.,* 2012) pero debido a los elevados niveles de expresión de *RHO* en los fotorreceptores, su silenciamiento y reemplazo a niveles suficientes siguen suponiendo un reto en la actualidad (Trapani *et al.,* 2015).

En relación con el establecimiento de posibles **correlaciones genotipo-fenotipo**, para poder orientar el pronóstico de los pacientes, algunos autores han tratado de relacionar la localización de la alteración dentro de la estructura proteica de la Rodopsina con el tipo de RP y grado de severidad (Sandberg *et al.,* 1995; Rakoczy *et al.,* 2011). En nuestro estudio los resultados no fueron concluyentes cuando se aplicaron sus conclusiones a nuestro espectro mutacional. El análisis de correlación genotipo-fenotipo para mutaciones en *RHO* se ve dificultado tanto por la variabilidad intra-e inter familiar, como por la baja proporción de pacientes con el mismo tipo de mutación. Nuestro trabajo, además de contar con uno de los espectros mutacionales más amplios descritos hasta la fecha para mutaciones en *RHO*, proporcionó extensa información clínica perteneciente a más de 100 pacientes de 42 familias con diagnóstico de adRP. El estudio de los datos oftalmológicos recopilados reveló que los pacientes con mutaciones en el gen *RHO*, en general, presentaban cambios en la retina consistentes en un fondo de RP típico, observándose movilizaciones de pigmento, vasos

estrechos y palidez del disco óptico y alteraciones en los registros de ERG. Además, en nuestro grupo de estudio, se estableció una media de edad de abolición del ERG de 38 años. Por otro lado, se analizó específicamente la correlación genotipo-fenotipo entre los pacientes que presentaban la mutación p.Pro347Leu con respecto a los que mostraron el resto de mutaciones en el gen *RHO* y se observaron diferencias estadísticamente significativas entre ambos grupos, en términos de edad al diagnóstico, inicio de ceguera nocturna, reducción del campo visual y pérdida de la agudeza visual. De esta forma, los pacientes con la mutación p.Pro347Leu presentaron un fenotipo de RP difusa con inicio más temprano y curso severo con escasa variación inter e intra-familiar, como también ha sido descrito anteriormente para esta variante en otras poblaciones (Berson *et al.,* 1991; Oh *et al.,* 2003). Por lo tanto esta es una de las escasas mutaciones en las que se puede proporcionar un pronóstico a los pacientes además de permitir centrar el diagnóstico, pronóstico y asesoramiento genético y reproductivo.

A lo largo de 23 años de estudio, desde 1991 hasta la publicación del artículo, se utilizaron diferentes herramientas clásicas de detección molecular para el estudio de las adRP y en concreto para el gen *RHO*: i) SSCP o DGGE y posterior secuenciación en muestras con patrón alterado, ii) secuenciación Sanger y iii) un *microarray* de genotipado específico para adRP que permite analizar hasta 131 mutaciones previamente descritas en *RHO*. En el momento de la publicación de este trabajo consideramos que el *microarray* era la técnica más apropiada para el cribado de estas familias debido a que presentaba valores de sensibilidad y especificidad adecuados, 94% y 100% respectivamente, (Blanco-Kelly *et al.,* 2012) y adicionalmente permitía cribar 283 mutaciones conocidas en otros 15 genes asociados a adRP.

Por lo tanto, considerando únicamente técnicas convencionales se propuso como algoritmo diagnóstico más efectivo para familias con adRP el uso del *microarray* específico, seguido del estudio mediante secuenciación Sanger de *RHO* para detectar mutaciones nuevas.

Panel de genes para diagnóstico molecular en adRP

Como consecuencia del desarrollo de la tecnología de *NGS* y su implementación en nuestro servicio, se pudo modificar posteriormente el algoritmo diagnóstico para las adRP. En este sentido, la secuenciación masiva dirigida de los genes relacionados con enfermedades muy heterogéneas como las DR parece ser la aproximación más rentable para el diagnóstico molecular de pacientes con adRP (Bowne *et al.,* 2011; Bernardis *et al.,* 2016). Partiendo de esta premisa, nuestro grupo desarrolló una estrategia basada en el enriquecimiento selectivo y secuenciación de 73 genes y regiones implicados en RP y LCA que se aplicó a una cohorte de 59 pacientes diagnosticados clínicamente de adRP en familias previamente cribadas y no

caracterizadas por técnicas convencionales. Los resultados de este estudio mostraron que el panel de genes era una herramienta efectiva con una tasa diagnóstica del 27%.

Los trabajos publicados de aplicación de *NGS* en pacientes con adRP han mostrado una gran variabilidad en su capacidad para detectar mutaciones, entre el 23 y 86% (Bowne *et al.,* 2011; Audo *et al.,*. 2012; Eisenberger *et al.,* 2013; Glöckle *et al.,* 2014; Daiger *et al.,* 2014), esto probablemente es debido a diferencias entre estos estudios en relación al tamaño de la cohorte estudiada, el número de genes incluidos en los diferentes paneles y principalmente, a los estudios moleculares previos realizados en los pacientes. Los casos seleccionados para nuestro estudio habían sido cribados mayoritariamente mediante el *array* de genotipado previamente descrito, y la secuenciación Sanger de *RHO* y otros genes más comunes asociados a adRP, como *PRPH2* (Trujillo *et al.,* 1998), *PRPF31* (Martínez-Gimeno *et al.,* 2003) y exones concretos de los genes *C1QTNF5, NR2E3* (Blanco-Kelly *et al.,* 2016)*, PRPF3, PRPF8* (Martínez-Gimeno *et al.,* 2003), *RP1* (Gamundi *et al.,* 2006) y *SNRNP200* entre otros, lo que probablemente subestimó la tasa de detección de nuestra estrategia para el análisis de casos *naive*. A pesar de ello, la tasa de diagnóstico con esta aproximación de secuenciación dirigida fue más elevada que la obtenida globalmente con el *microarray* de genotipado en nuestra cohorte de adRP, estimada entre el 15% y el 20 % (Blanco-Kelly *et al.,* 2012; Fernandez-San Jose *et al.,* 2014). Además, explica que exista un sesgo en los genes detectados por esta estrategia encontrándose principalmente implicado el gen *RP1* en el 7% de los casos. En este gen se identificaron mutaciones nuevas, siendo tres de ellas de tipo *LOF* (p.Gly314Argfs*10, p.Glu661* y p.Tyr915*) y una cuarta mutación *missense* (p.Arg1443Gln) que se consideró como patogénica al haber cosegregado con la enfermedad en la familia con un patrón de herencia autosómico dominante, haber sido predicha como patogénica mediante análisis *in silico*, ser excluida en 300 cromosomas de controles españoles y localizarse en el *cluster* de mutaciones asociadas a adRP. Adicionalmente, en los genes *PRPF31, PRPH2 y SNRNP200* se encontraron mutaciones responsables en el 3% de nuestras familias para cada uno de ellos.

Este panel de 73 genes relacionados con RP y LCA se desarrolló en nuestro laboratorio con un diseño que asegurase una alta cobertura de las regiones diana (99,1%) y se secuenció a elevada profundidad (>500x) con el fin de minimizar los errores de secuenciación descritos para las nuevas tecnologías de NGS (Sims *et al.,* 2014). Este panel pudo ser utilizado para analizar un total de 176 familias con distintos tipos de DR (59 adRP, 47 arRP, y 70 LCAs) demostrando una elevada sensibilidad y especificidad al ser validado en muestras que presentaban polimorfismos o alelos recesivos en heterocigosis ya conocidos (Perez-Carro et al. 2016) mostrándose por tanto como una técnica fiable para cambios de una sola base. En esta

misma publicación nuestro grupo pudo evaluar la aplicación del panel en familias diagnosticadas de arRP y sRP demostrando ser también una herramienta efectiva para su diagnóstico molecular, con una tasa de detección del 57% (Perez-Carro *et al.,* 2016).

Además de poder utilizar este panel en distintas formas de DR, otra de las ventajas de este diseño es que utiliza una estrategia de análisis libre de hipótesis, lo que permitió reclasificar genéticamente un total de tres familias en las que se habían descartado primero variantes en genes dominantes. En dos de ellas, los casos índice eran mujeres portadoras afectadas con mutaciones nuevas en los genes *RPGR* y *RP2*, ligados al cromosoma X y síntomas severos de RP. Estos hallazgos no han sido sorprendentes puesto que se ha descrito previamente que el 8,5% de las familias con diagnóstico inicial de adRP y mujeres portadoras afectadas, presentan mutaciones en genes ligados al cromosoma X habiendo sido descrita una subestimación de la frecuencia de xlRP vs adRP en algunas poblaciones (Daiger *et al.,* 2008; Bowne *et al.,* 2011; Churchill *et al.,* 2013). Por último, una tercera familia, RP-1875, fue finalmente diagnosticada de DCB, con un patrón de herencia pseudodominante. El caso índice presentaba dos mutaciones en heterocigosis en el gen *ABCA4* y se identificó únicamente uno de los cambios en una tía afectada. Dada la frecuencia de alelos mutados en *ABCA4* en nuestra población (Riveiro-Alvarez *et al.,* 2013), este hallazgo no es inusual pudiendo encontrarse familias que presenten heterogeneidad genética intrafamiliar (Sanchez-Alcudia *et al.,* 2016). Además el panel detectó dos mutaciones en los genes *CRX* y *PRPF31* previamente cribadas con el *microarray* de genotipado, lo que ha mostrado dos falsos negativos para esa técnica. Estos datos coinciden con la sensibilidad del 95% que ya había sido descrita con anterioridad al evaluar este *microarray* por nuestro grupo (Blanco-Kelly *et al.,* 2012) así como en el primer artículo expuesto en este trabajo (Fernandez-San Jose et al., 2014).

Por otro lado, el desarrollo de las técnicas de *NGS* ha venido acompañado de nuevos desafíos en la interpretación de la patogenicidad de las variantes detectadas; por ello, es importante conocer las características clínicas y genéticas de la enfermedad en estudio y así poder reducir al máximo el número de variantes candidatas para su evaluación. A diferencia de las formas recesivas, en las que algunas mutaciones pueden presentar frecuencias más elevadas en algunas poblaciones, p.e. *ABCA4* (Riveiro-Alvarez *et al.,* 2013) en el caso de adRP, los cambios responsables presentan una frecuencia extremadamente baja (Daiger *et al.,* 2014). Por ello se adaptó nuestro el algoritmo bioinformático de priorización de variantes por frecuencia alélica al análisis específico de formas dominantes utilizando valores de MAF<0.001, lo que permitió disminuir el número de *VUS* a analizar. Además, todas ellas se buscaron tanto en bases de datos de frecuencias alélicas como en controles sanos españoles y se analizó el

efecto sobre la proteína del cambio de nucleótido utilizando también en los casos en los que no había literatura al respecto, programas predictores de patogenicidad. Por otro lado se realizaron análisis de co-segregación para evaluar la implicación de las variantes, aunque estos resultados tienen limitaciones en su interpretación en familias adRP, ya que pueden existir mutaciones *de novo* como la mutación p.Met44Thr en *RHO* presente en el caso esporádico RP-0041 de nuestra serie (Reig *et al.,* 1994; Fernandez-San Jose *et al.,* 2014) y otras descritas por otros autores (Davies *et al.,* 2012; Shanks *et al.,* 2013); casos de penetrancia incompleta (Martínez-Gimeno *et al.,* 2003; Gamundi *et al.,* 2006) y casos de variabilidad intrafamiliar, tan frecuentemente asociados a formas dominantes de RP. Este es el caso de la familia RP-1728, descrita en este artículo, que presentaba una variante nueva en el gen *GUCA1B* que durante la segregación familiar fue detectada en un miembro asintomático en el momento del estudio, no pudiéndose descartar la mutación como causante de la enfermedad por haberse descrito otros casos de penetrancia incompleta en este gen (Sato *et al.,* 2005). Además, en la adRP la aparición de síntomas suele ser algo más tardía que en otras formas de distrofia de retina por lo que no siempre los síntomas han debutado en algunos miembros de la familia en el momento del estudio, lo que dificulta esta interpretación de patogenicidad o de penetrancia incompleta para algunas variantes nuevas. En estos casos, el posterior seguimiento de los pacientes resulta fundamental para poder establecer la causalidad de las variantes.

Otra de las ventajas de la tecnología de *NGS* es que permite un posible reanálisis de los datos a medida que mejoran los algoritmos bioinformáticos y aumenta la información de las bases de datos. De este modo, los datos de secuencias generados con este panel de genes han sido analizados recientemente utilizando un nuevo *pipeline* implementado en nuestro laboratorio que incluye no sólo un reanálisis de variantes tipo *SNVs* con nuevos algoritmos más sensibles sino además el análisis de *CNVs* utilizando datos de cobertura de los distintos exones. Destacar que de esta manera se han podido detectar nuevas *CNVs* en *PRPF31*, que han sido validadas mediante *MLPA* y/o *aCGH* (Martin-Merida *et al.,* 2017) o una deleción del exon 5 en *RHO* en una familia con adRP que había sido analizada en paralelo a nuestro trabajo (de Sousa *et al.,* 2015). Estos hallazgos son similares a los publicados por nuestro grupo con familias arRP en el que se demostró la utilidad de este panel para la detección de *CNVs* en el 4% de los casos siendo responsables los genes *USH2A* y *EYS* (Perez-Carro *et al.,* 2016). Otros trabajos recientes también identifican deleciones genómicas en distintas formas de DR, estimándose entre el 4% y 18% el número de los casos con DR que presentan mutaciones del tipo *CNV* (Khateb *et al.,* 2016; Bujakowska *et al.,* 2017; Van Cauwenbergh *et al.,* 2017).

Tras la aplicación de esta tecnología de *NGS*, aún permanece sin caracterizar un porcentaje elevado de los casos. En la actualidad se estima que un 40% de los pacientes con DR sigue sin resolverse (Audo *et al.,* 2012; Shanks *et al.,* 2013) lo que puede ser debido a la presencia de variantes en: i) regiones no codificantes, bien regiones intrónicas y/o reguladoras; ii) regiones repetitivas, que son difíciles de capturar con estas técnicas, como sucede con la región *ORF15* en el gen *RPGR*; iii) genes recientemente identificados como *ADIPOR1*, *ARL3, PRPF4 HK1* y SSP2 (Chen et al. 2014; Sullivan *et al.,* 2014; Liu *et al.,* 2015; Ganesh *et al.,* 2011; Strom *et al.,* 2016; Zhang *et al.,* 2016); iv) nuevos genes aún por descubrir y v) variantes estructurales tales como inversiones, duplicaciones y deleciones que pueden ser difíciles de resolver con tecnología *NGS.*

En el caso de las variantes no codificantes, normalmente los diseños de paneles dirigidos no suelen incluir las regiones intrónicas profundas de los genes diana debido a limitaciones de cobertura con relación a su coste-efectividad. Sin embargo, se han descrito variantes intrónicas en distintos genes asociados a DR como *USH2A* (Garcia-Garcia *et al.,* 2014), *ABCA4* (Zernant *et al.,* 2014) y *OFD1* (Webb *et al.,* 2012), entre otros, que conducen a la inclusión de pseudoexones mediante la activación de sitios crípticos de *splicing.* En nuestro panel se incluyeron algunas de las regiones intrónicas que habían sido asociadas a DR en el momento del diseño (Perez-Carro *et al.,* 2016). Mecanismos similares que conducen a la alteración del *splicing* o la desregulación de sitios de unión para factores de transcripción pueden estar causados por algunas variantes sinónimas, sin embargo, éstas no suelen ser consideradas durante el análisis y filtrado de variantes a pesar de las crecientes evidencias de su implicación en más de 50 enfermedades asociadas (Hunt *et al.,* 2014). Tampoco se suelen analizar regiones reguladoras tales como *enhancers*, *miRNAs* y/o sus regiones diana, que también pueden estar implicadas en algunas formas de DR, como ya ha sido descrito previamente para el *mir-204* en una familia con adDR y coloboma bilateral (Conte *et al.,* 2015). Se espera que en el futuro el análisis de estas regiones no codificantes en genes asociados a adRP aumente el rendimiento en la caracterización de nuestros pacientes.

Secuenciación de exoma completo en familias con adRP

Se estima que el 50% de los casos de adRP presentan mutaciones en genes aun no descubiertos como asociados a la patología (Daiger *et al.,* 2013), lo que sugiere la existencia demutaciones en otros genes que aún no han sido identificadas (Kannabiran *et al.,* 2012). Prueba de ello es la identificación del locus RP63 detectado mediante análisis de ligamiento, para el cual aún no ha sido identificado el gen causal. La aplicación de otras aproximaciones de *NGS* basadas en *WES* o, más aún en *WGS* permitirá la identificación de nuevos genes o nuevas

variantes potencialmente patogénicas en regiones no codificantes. En paralelo al desarrollo del panel de genes mediante *NGS*, nuestro grupo tuvo la oportunidad de adquirir experiencia en el manejo de la secuenciación de exoma completo con el fin de tratar de identificar nuevos genes aplicando dicha tecnología a un grupo de siete familias con diagnóstico inicial de adRP permitiendo caracterizar el 71% de ellas.

Nueva asociación fenotípica de PRPS1 con una forma sindrómica de RP

Esta tecnología *WES* nos permitió identificar una nueva mutación en el exón 1 de gen *PRPS1*, p. Ser16Pro, ligado al cromosoma X, que provocaba un déficit de la enzima PRS-I en las mujeres de una familia que presentaba retinosis pigmentaria típica ó sectorial y otras manifestaciones neurológicas con una edad de inicio, presentación y la gravedad del fenotipo muy variables, ampliando así el espectro fenotípico de este gen y permitiendo su inclusión en posteriores estudios genéticos a pacientes con RP.

Diferentes mutaciones en el gen *PRPS1* se han relacionado con distintas enfermedades como el Síndrome de Arts, CMTX5, DFN2 y el Síndrome de Superactividad de PRS-I, todas ellas asociadas a distintas manifestaciones clínicas que en ningún caso incluían la RP. Las mutaciones en *PRPS1* son raras y pueden dar lugar a un aumento o disminución de la actividad de la enzima PRS-I, que se expresa de forma ubicua y tiene un papel esencial en el metabolismo de nucleótidos, catalizando la síntesis del sustrato para la síntesis de nucleótidos de purina, piridina y pirimidina (Roessler *et al.,* 1990). Aunque otros autores han descrito con anterioridad que las mujeres portadoras de mutaciones en *PRPS1* pueden presentar disminución en la actividad del enzima PRS-I y un fenotipo alterado (Rosenberg and Chutorian, 1967; Pauli*,* 1984; de Brouwer *et al.,* 2007; Synofzik *et al.,* 2014), hasta la fecha no se había publicado ningún caso de mujeres con un fenotipo tan complejo y grave como el observado en esta familia. Las pacientes mostraron un fenotipo con características similares al de síndrome de Arts y CMTX5, con atrofia óptica como primer síntoma, seguido de RP con diferentes manifestaciones neurológicas, como pérdida auditiva, discapacidad intelectual, ataxia y neuropatía periférica en grado variable. Estos hallazgos son consistentes con la creciente evidencia de fenotipos intermedios en síndromes asociados al PRS-I, recientemente descritos por varios autores (Moran *et al.,* 2012; Park *et al.,* 2013; Synofzik *et al.,* 2014; Al-Maawali *et al.,* 2015; Gandía *et al.,* 2015). Recientemente Synofzik y cols. describieron varias presentaciones fenotípicas dentro de una misma familia, incluyendo un varón con un fenotipo intermedio entre CMTX5 y Síndrome de Arts y una mujer portadora afectada de DFN2 debido a la inactivación sesgada del cromosoma X (Synofzik *et al.,* 2014). Estos autores observaron en la mujer portadora una correlación entre la actividad residual enzimática, el grado de

inactivación del cromosoma X y el fenotipo. Además, sostenían que la localización de la mutación y la actividad enzimática residual eran los principales determinantes de las manifestaciones fenotípicas variables entre varones y mujeres (Synofzik *et al.,* 2014).

En nuestro trabajo, se observó una correlación similar en el caso índice de nuestra familia que presentaba un fenotipo de mayor gravedad. En el caso de la madre y hermana, la inactivación del cromosoma X no estaba sesgada y el grado de déficit de PRS-I que presentaban sólo se pudo correlacionar con la edad de inicio de los síntomas oftalmológicos, pero no con la presentación ni la gravedad del fenotipo. Esta falta de correlación no es sorprendente, pues la inactivación de X, ya sea al azar o sesgada, es tejido-específica y por tanto el estado de inactivación en un determinado tipo de tejido como el observado en los leucocitos, en nuestro estudio, puede no ser representativo del estado del sistema nervioso central y periférico (Synofzik *et al.,* 2014; Van den Veyver, 2001) que son los principalmente afectados en los síndromes de déficit de PRS-I. Por ello, debido a la gran variabilidad en la expresión de la enfermedad en nuestra familia, es posible que la inactivación del cromosoma X no se encontrara sesgada en esos tejidos. Otra posibilidad para explicar las diferencias en la expresividad y severidad de los síntomas en nuestra familia es que otras isoformas del enzima, como PRS-II estén compensando la función enzimática de PRS-I, tal y como han sugerido otros autores (de Brouwer et al. 2010), y por lo tanto la síntesis del sustrato para la formación de nucleótidos no se vería afectada de forma crítica. Estos mismos autores también han sugerido que existe una relación entre la localización de la mutación en el gen *PRPS1* y las diferentes manifestaciones sistémicas, siendo más graves los síndromes causados por mutaciones que afectan a los sitios activos y alostéricos del enzima PRS-I. Simultáneamente al presente trabajo, Al-Maawali y sus colaboradores ampliaron el fenotipo *PRPS1* a distrofia de retina y diabetes insípida en una familia con dos varones afectados por *LCA* con otras manifestaciones y con mujeres portadoras asintomáticas (Al-Maawali *et al.,* 2015). La mutación p.Arg196Trp responsable de este nuevo fenotipo se localiza próxima al sitio de unión a ATP de PRS-I. En el caso de nuestra familia, el cambio hallado, p. Ser16Pro está localizado en el dominio N-terminal de la proteína, en una región que nunca se había encontrado alterada hasta la fecha y alejada de los sitios alostéricos y activos de la enzima que están afectados en el síndrome de Arts, CMTX5 y en este nuevo fenotipo recientemente asociado a distrofia de retina. Esto podría demostrar, una vez más, que la localización de la mutación puede explicar la diversidad de fenotipos hallados asociados a la alteración genética de esta proteína (de Brouwer *et al.*, 2010; Synofzik *et al.,* 2014; Gandía *et al.,* 2015).

Por otro lado, aunque en los análisis de expresión génica realizados en nuestro trabajo en muestras procedentes de linfocitos de sangre periférica no mostraron diferencias en los niveles transcripcionales de *PRPS1* entre los portadores y no portadores de la mutación y no se encontraron transcritos aberrantes adicionales, no se ha podido descartar la presencia de una isoforma defectuosa de *splicing* como predijeron los análisis *in silico* realizados en este trabajo, que pudiera haberse degradado, y por tanto podría no haberse detectado en el ensayo de RT-PCR. Tampoco se ha podido descartar que otros genes o factores epigenéticos puedan contribuir a la complejidad y gravedad de los fenotipos asociados al déficit de PRS-I. En este trabajo tampoco se ha podido descartar un patrón de herencia dominante ligada al X para esta mutación en concreto, con un efecto letal en los hemicigotos, debido a la falta de varones afectados en la familia. Por último, debido la gran variabilidad que presenta el fenotipo descrito y a que ha quedado demostrado que las mujeres portadoras de mutaciones en *PRPS1* pueden presentar un fenotipo tan severo como los varones, este gen ligado al cromosoma X debería considerarse en el estudio de cualquier paciente que presente DR y/o atrofia óptica.

Secuenciación exómica completa en familias con diagnóstico inicial de adRP

Como parte de este trabajo de colaboración internacional, se realizó un análisis de *WES* en otras seis familias con un diagnóstico inicial de adRP en las cuales se habían realizado sin éxito estudios moleculares previos. Utilizando este enfoque, pudimos caracterizar a cuatro de las seis familias y, en todas ellas, se hallaron mutaciones en genes conocidos (*PRPH2, PRPF31, RPGR* y *C1QTNF5)* con la reclasificación de tres de las familias, bien a a otras formas genéticas, como xlRP en la familia RP-0858, como a otros fenotipos o fenotípicas, como una degeneración retiniana de inicio tardío (*LORD*) en la familia RP-0911 y distrofia de conos-bastones, en la familiaRP-0107.

En el caso de la familia RP-0858, las siete mujeres portadoras de la mutación en *RPGR* mostraban un fenotipo de RP severo, sin variabilidad intra-familiar significativa comparada con los tres varones afectados, por ello se sospechó un modelo de herencia dominante. Este hallazgo junto con observaciones similares en el trabajo anterior con nuestro panel de genes (Fernandez-San Jose *et al.,* 2015), hacen que sea altamente recomendable el estudio de genes ligados al cromosoma X en todas las familias de RP con un aparente patrón de herencia autosómico dominante y que no presenten transmisión varón-varón, algo cada vez más común puesto que las familias constan de menos individuos.

Otro caso complejo al que la secuenciación exómica completa pudo dar respuesta, fue la familia RP-0911. Se encontró la mutación pSer163Arg en heterocigosis en el gen *C1QTNF5*

responsable de una forma extremadamente rara de DR autosómica dominante de inicio muy tardío denominada *LORD*. Esta mutación es hasta el momento la única descrita en este gen en menos de 15 familias familias diferentes (Jacobson *et al.,* 2001; Subrayan et al. 2005; Vincent *et al.,* 2012; Soumplis *et al.,* 2013) y está asociada a un fenotipo particular que en estadíos tempranos se caracteriza por problemas de adaptación a la oscuridad y depósitos tipo drusas en la región macular periférica, por lo que puede diagnosticarse erróneamente como degeneración macular relacionada con la edad (DMAE), y en estadíos tardíos como una retinosis pigmentaria (Kuntz *et al.,* 1996; Milam *et al.,* 2000; Soumplis *et al.,* 2013). Debido a los limitados datos clínicos disponibles en esta familia y a la falta de literatura relacionada con esta entidad en el momento en el que la familia fue evaluada por primera vez, LORD nunca fue considerado como posible causa y a los tres miembros afectados se les atribuyeron diferentes diagnósticos de RP, DMAE e incluso la sospecha de la coexistencia de ambas entidades. Una nueva reevaluación clínica de la familia, tras el resultado obtenido con *WES*, evidenció que algunos de los síntomas presentes en portadores eran consistentes con el fenotipo característico descrito anteriormente para esta mutación, mientras que otros como la neovascularización coroidea, no estaban presentes en el momento del diagnóstico.

El análisis del exoma completo también ayudó al diagnóstico preciso de la familia RP-0107, encontrándose la mutación p. Asp186Asn en *PRPH2* previamente descrita como asociada a una distrofia de conos autosómica dominante (Kitiratschky *et al.,* 2011). En nuestra familia todos los miembros afectados presentaron fondo de ojo y campos visuales compatibles con una RP con afectación macular, excepto un individuo que mostraba degeneración retiniana difusa. Debido a que fueron estudiados oftalmológicamente en las últimas etapas de la enfermedad, el fenotipo en ese momento parecía más compatible con una RP evolucionada, por lo que fueron diagnosticados clínicamente de adRP. Tras el hallazgo encontrado mediante *WES*, la familia fue reclasificada como distrofia de conos-bastones. Por otro lado, el cribado de *PRPH2* en esta familia realizado años atrás por DGGE no identificó la alteración susceptible de indicar mutación, por lo que este hallazgo representa un falso negativo para esta técnica.

En algunos de los casos reclasificados que hemos presentado, la información clínica disponible era escasa o estaba restringida a estadíos tardíos de la enfermedad, siendo esta limitación junto con la clínica solapante que presentan las diferentes formas de distrofias retina, las que provocan que su diagnóstico sea extremadamente complejo. Por lo tanto, el uso de un enfoque libre de hipótesis para la detección de mutaciones, como es la aproximación *WES*, ayuda a minimizar el impacto que tiene la disponibilidad de información clínica del paciente y de la familia sobre el éxito en el diagnóstico genético de la enfermedad.

En una última familia, RP-0777, el análisis de *CNVs* de los datos obtenidos mediante *WES* permitió detectar una gran deleción que abarcaba el gen *PRPF31* además de los genes *OSCAR*, *NUDFA3* y *TFPT*. El gen *PRPF31* es uno de los más frecuentemente alterados en adRP, representando entre el 7 y el 10% de todos los casos y donde las deleciones completas o parciales del gen son responsables de alrededor del 2-3% de los pacientes (Daiger *et al.,*. 2008; Martin-Merida *et al.,* 2017). Curiosamente, a pesar de que las técnicas de *NGS* han sido ampliamente utilizadas para la caracterización de familias con distrofia de retina en los últimos 5 años, hasta la fecha de publicación del artículo de nuestro grupo en 2015, muy pocos trabajos describían la detección de grandes deleciones asociadas a DR utilizando datos de secuenciación (Eisenberger *et al.,* 2013; Nishiguchi *et al.,* 2013), por lo que nuestro estudio apoyó la viabilidad de detectarlas ampliando así su potencial en el diagnóstico de estas enfermedades. Posteriormente otros autores han utilizado con éxito datos de *WES* detectando *CNVs* en el 10 y 18% de sus pacientes (Khateb *et al.,* 2016; Bujakowska *et al.,* 2017).

Finalmente, dos familias no pudieron ser caracterizadas mediante *WES* y tampoco se evidenciaron posibles variantes patogénicas en genes asociados a DR o en nuevos genes. Esto no resulta extraño cuando aplicamos *WES* en formas dominantes, que son extremadamente complejas tanto por su contexto de heterocigosidad como por la presencia de penetrancia incompleta (Martínez-Gimeno *et al.,* 2003; Gamundi *et al.,* 2006). En general, para aumentar la posibilidad de éxito sería necesario analizar familias altamente informativas, con un elevado número de afectos y sanos presentes en distintas fratrias, con el fin de realizar análisis de segregación que sean concluyentes en las variantes potencialmente candidatas.

La dificultad que existe para identificar nuevas causas de adRP se evidencia observando el mayor número de genes descritos asociados con las formas recesivas en comparación con formas dominantes (58 vs 27 genes, respectivamente). Hay que tener en cuenta que en la era de la NGS, en los últimos seis años se han identificado 53 nuevos genes relacionados con DR mediante *WES/WGS. D*e ellos, únicamente 8 se asocian a herencia dominante y 45 a formas recesivas. Es de destacar que el uso combinado de *WES* con estrategias de mapeo de homozigosidad o análisis de ligamiento permite aumentar la tasa de éxito en la identificación de nuevos genes. Así, en este periodo fueron identificados 22 genes recesivos y dos nuevos genes asociados a formas dominantes de DR (Broadgate *et al.,* 2017). Esta estrategia de análisis de ligamiento que fue utilizada con éxito en nuestro grupo para la identificación de mutaciones en genes asociados a formas dominantes como *IMPDH1* y *GUCA1A* (Jordan *et al.,* 1993; Kamenarova *et al.,* 2013) podría ser también válida para mejorar el rendimiento del análisis *WES* mediante la priorización de variantes en regiones de

ligamiento obtenidas mediante *arrays* de genotipado de genoma completo. Un ejemplo de su utilidad fue la identificación de *IMPG1* como nuevo gen asociado a distrofias maculares viteliformes (Manes *et al.,* 2013).

Los resultados del presente estudio, donde la mayor parte de genes detectados eran conocidos, indican que *WES* puede ser una herramienta diagnóstica eficaz y adecuada para su implementación en rutina clínica (Corton *et al.,*. 2013; Riera *et al.,* 2017), En relación con esta aplicación diagnóstica, tanto la técnica de *WES* como los paneles de genes presentan tasas de identificación de mutaciones similares, en torno al 60% (Tiwari *et al.,* 2016; Farrar *et al.,* 2017), y su uso conlleva ventajas e inconvenientes. Entre las ventajas de los paneles dirigidos respecto al *WES*, destaca su menor coste, tanto en reactivos de secuenciación como a nivel bioinformático pues su implementación metodológica no requiere ordenadores o servidores tan potentes como los necesarios en el análisis de *WES*. Además requiere menor tiempo de análisis puesto que la interpretación de variantes está acotada a los genes incluidos en el panel. Por otro lado, con los paneles se pueden obtener profundidades de lectura mayores de forma coste-efectiva, permitiendo obtener mayores coberturas de las regiones diana y mayor uniformidad entre las muestras (Consugar *et al.,* 2015), lo que permite la detección más fiable de algunos tipos de variantes como *indels*, mosaicos y *CNVs*. Por el contrario, entre las limitaciones de los paneles destaca la necesidad de constantes actualizaciones de los sistemas de captura debiendo incluir genes a medida que éstos se van identificando. Sin embargo, con *WES* las actualizaciones se realizan de forma más sencilla modificando los algoritmos de análisis incluyendo los nuevos genes a modo de paneles virtuales. Además, *WES* permite explorar adecuadamente el papel de posibles genes modificadores que pueden ser importantes para explicar la posible penetrancia incompleta y/o en la modulación del fenotipo, como se ha descrito para *PRPF31* y *PRPH2* (Rose *et al.,* 2016; Conley *et al.,* 2017), mientras que con los paneles dirigidos esto sólo es posible si se evalúan estos efectos modifcadores únicamente en genes conocidos.

Del mismo modo, si comparamos las estrategias de *WES* con la WGS, las limitaciones en cuanto a precio, tiempo de análisis y almacenamiento, se acrecientan. Además, se estima que el 85% de las mutaciones causantes de enfermedad se encuentran en el exoma o en los sitios de empalme que flanquean los exones, por lo que *WES* sería más eficiente para encontrar variantes raras (Ng *et al.,* 2009). Sin embargo, cada vez hay más evidencias de la implicación de regiones reguladoras e intrónicas profundas con un papel relevante en la enfermedad que no suelen ser incluidas en las estrategias de secuenciación dirigidas y que podrían ser potencialmente identificados mediante *WGS*. Además, esta tecnología no requiere

un paso de enriquecimiento que puede sesgar la secuenciación dando lugar a regiones pobremente enriquecidas en zonas repetitivas como sí sucede con los sistemas de captura de secuencia utilizados en los *WES* y en muchas de las estrategias de paneles dirigidos. Estas ventajas, junto con los avances en la potencia informática y conocimiento en el genoma humano, hace que la introducción gradual de *WGS* para el diagnóstico clínico, sea una cuestión de probablemente poco tiempo. En la actualidad, basándonos en nuestra experiencia de los últimos años en *NGS*, y en consonancia como lo descrito por otros autores (Audo *et al.,* 2012; Consugar *et al.,* 2015), proponemos iniciar las pruebas de diagnóstico de los pacientes con sospecha de adRP con secuenciación dirigida, debido a las ventajas metodológicas que presenta. Tras este análisis, en los casos negativos proponemos utilizar *WES*/*WGS*, como segundo paso para la identificación de nuevos genes en un contexto de investigación (Figura S2). Además, como existe una alta incidencia de xlRP entre las familias inicialmente clasificadas como dominantes (Churchill *et al.,* 2013), y las mutaciones en *ORF15* son responsables de hasta el 60% de xlRP (Vervoort *et al.,* 2000), proponemos analizar esta región mediante secuenciación Sanger, puesto que no puede ser totalmente cubierta por los actuales métodos de *NGS* debido a su naturaleza repetitiva, en todas las familias que no muestren transmisión varón-varón, independientemente de la sintomatología o el número de mujeres afectadas, y así poder descartar una posible herencia ligada al cromosoma X.

En resumen, las DR son un grupo de enfermedades fenotípica y genéticamente muy heterogéneas que suponen un desafío para su diagnóstico basándose únicamente en el fenotipo de la enfermedad. Por ello, las aproximaciones genéticas basadas en *NGS* desempeñan un papel crucial, no solo para apoyar el diagnóstico clínico, pronóstico, asesoramiento genético y reproductivo, sino también para poder estratificar los casos para posibles intervenciones terapéuticas dirigidas al defecto genético específico.

CAPÍTULO VI

Conclusiones

Conclusiones

1. Las mutaciones en el gen *RHO* representan la principal causa de adRP en población española, con una prevalencia del 21%. La mutación más frecuente es p.Pro347Leu, representando el 4,5% de los casos de adRP. Se ha podido establecer una correlación genotipo-fenotipo para esta mutación que se asocia a un fenotipo de inicio más temprano y curso más grave, en comparación con otras variantes detectadas en dicho gen.

2. El algoritmo propuesto para el diagnóstico de familias con adRP, considerando técnicas convencionales, consta del uso de un *microarray* de genotipado específico de adRP, que adicionalmente permite el cribado de mutaciones conocidas en otros genes, seguido del estudio mediante secuenciación Sanger de los genes más frecuentes asociados a adRP, en los casos negativos, para detectar mutaciones nuevas no incluidas en el array.

3. Con la tecnología de *NGS* basada en un panel de 73 genes evaluada en este trabajo para el análisis de adRP, se han caracterizado 16 de 59 familias, obteniendo una tasa de diagnostico del 27%, mostrando un incremento respecto a la tasa diagnóstica del 20% del *array* de genotipado específico para adRP.

4. La estrategia de secuenciación dirigida ha demostrado ser efectiva para el diagnóstico de pacientes con adRP, identificándose un total de 17 mutaciones de las cuales 11 (65%) son nuevas, aportando información relevante sobre las mutaciones encontradas, permitiendo proporcionar un diagnóstico preciso y un adecuado asesoramiento genético a los pacientes y sus familiares.

5. La tasa diagnóstica de *WES* en familias con diagnóstico inicial de adRP fue del 71%, caracterizándose cinco de las siete familias analizadas. Además se han podido reclasificar clínica y /o genéticamente cuatro de las familiasestudiadas.

6. El estudio mediante exoma completo ha permitido la identificación de una mutación nueva en el gen *PRPS1*, asociándose por primera vez dicho gen a mujeres con distrofia de retina y estableciéndose una nueva asociación que amplía así el espectro de fenotipos asociados a dicho gen.

7. El análisis bioinformático de los datos generados por el exoma ha permitido identificar una gran deleción en el gen *PRPF31* demostrando el potencial de esta tecnología para la detección de reordenamientos genómicos.

8. Basándonos en los resultados obtenidos en este estudio, mediante tecnología de secuenciación masiva se propone un algoritmo actualizado para la caracterización molecular de los casos de adRP. Este consiste en la aplicación de secuenciación dirigida como primer abordaje diagnóstico y posterior secuenciación Sanger de la región *ORF15* en familias sin evidencia de transmisión varón-varón. En una segunda fase, el uso de la secuenciación del exoma completo en casos negativos representa un abordaje óptimo para identificar nuevos genes asociados a distrofias de retina.

9. Tomados en conjunto los datos obtenidos del panel dirigido y de la técnica WES, se han reclasificado clínica y/o genética un total de ocho familias españolas diagnosticadas inicialmente de adRP , pudiendo definir mejor el diagnostico, dar un adecuado asesoramiento genético y reproductivo y permitiendo su posible inclusión en futuras aproximaciones terapéuticas.

CAPÍTULO VIII

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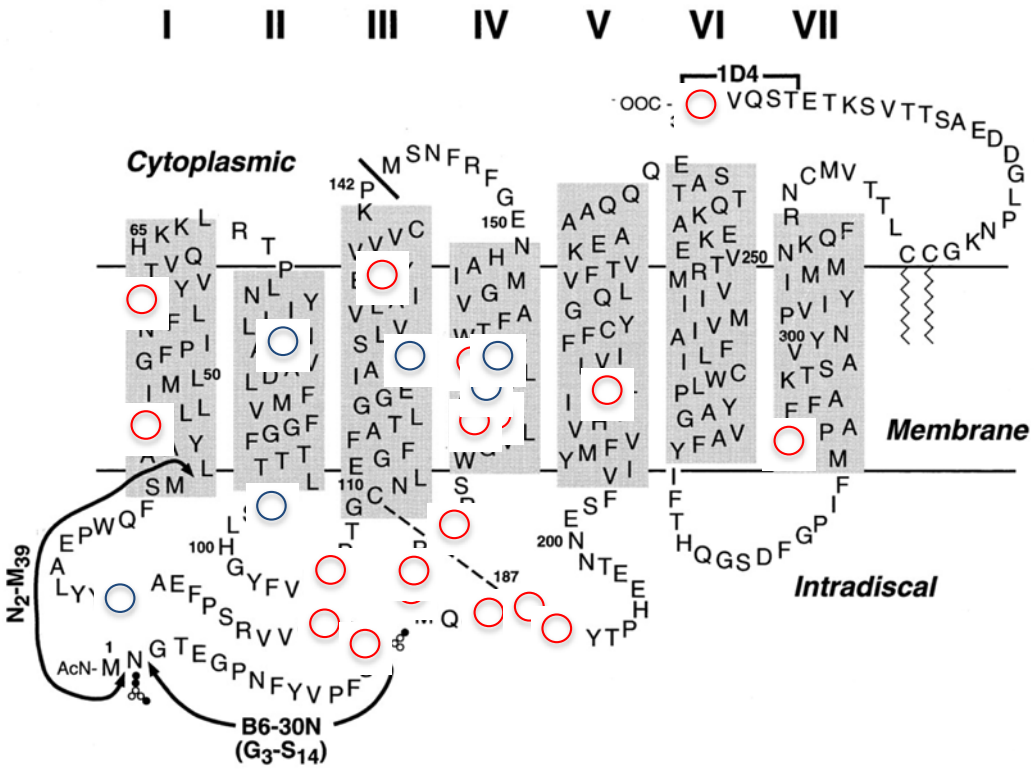
Anexo I

Figura S1. Esquema de la estructura secundaria de la proteína rodopsina. Localización de los aminoácidos afectados por las mutaciones detectadas en en el gen *RHO*.



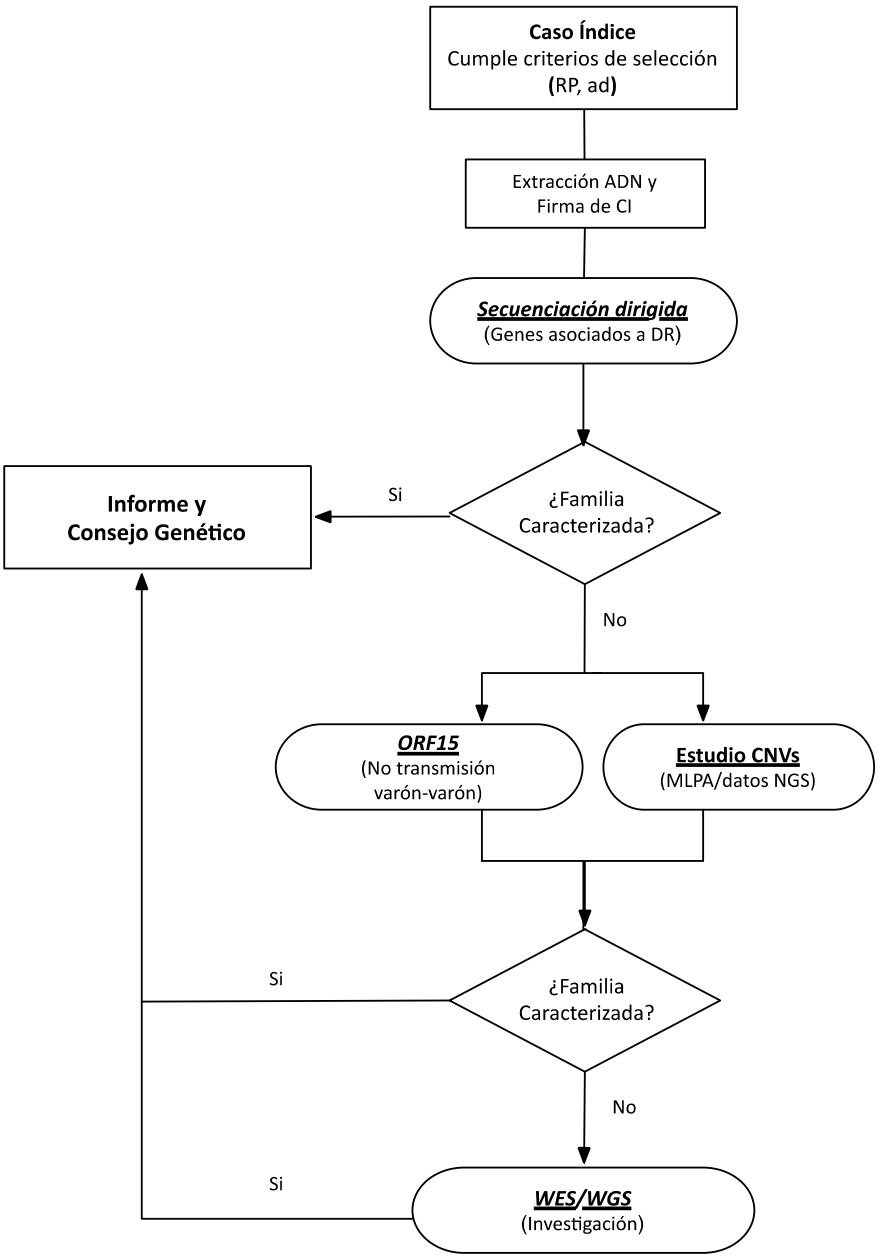
MUTACIONES DESCRITAS

p.Asn15Ser	→ 1 familia	p.Gly106Arg	→ 1 familia	p.Pro170Arg	→ 1 familia	p.Glu181Lys	→ 1 familia	p.Asp190Tyr	→ 2 familia
p.Thr17Met	→ 1 familia	p.Arg135Trp	→ 1 familia	p.Pro171Leu	→ 1 familia	p.Gly182Ser	→ 2 familia	p.Pro215Leu	→ 1 familia
p.Met44Thr	→ 1 familia	p.Arg135Leu	→ 2 familia	IVS2 as A-G -2	→ 1 familia	p.Ser186Pro	→ 1 familia	p.Thr289Pro	→ 1 familia
p.Thr58Arg	→ 2 familia	p.Ala164Glu	→ 2 familia	p.Tyr178Cys	→ 1 familia	p.Gly188Arg	→ 1 familia	p.Pro347Leu	→ 8 familia

MUTACIONES NUEVAS

p.Gln28His	→ 1 familia
p.Leu79Pro	→ 1 familia
p.Thr97Ile	→ 1 familia
p.Trp126*	→ 1 familia
p.Met163Thr	→ 1 familias
p.Cys167Tyr	→ 2 familias
p.Gly182Val	→ 1 familia

Figura S2. Algoritmo diagnóstico propuesto para familias con adRP mediante NGS.



Leyenda: RP, Retinosis Pigmentaria; ad: autosómico dominante; CI: Consentimiento informado

Anexo II

Otras publicaciones derivadas de esta tesis doctoral

I Martin-Merida, R Sanchez-Alcudia, **P Fernandez-San Jose**, F Blanco-Kelly, R Perez-Carro, M Corton, L Rodriguez J. da Silva, B Almoguera, B Garcia-Sandoval, M Isabel Lopez-Molina, A Avila-Fernandez, M Carballo, C Ayuso. Analysis of the *PRPF31* gene in Spanish autosomal dominant Retinitis Pigmentosa patients: a novel genomic rearrangement. Invest Ophthalmol Vis Sci. 2017 Feb 1;58 (2):1045-1053.

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